

# REPRODUCTION IN DOMESTIC ANIMALS

THIRD EDITION

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# REPRODUCTION IN DOMESTIC ANIMALS

THIRD EDITION

EDITED BY

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ACADEMIC PRESS New York San Francisco London 1977

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ACADEMIC  
111 Fifth Avenue, New York, New York 10003

*United Kingdom Edition published by*  
ACADEMIC PRESS, INC. (LONDON) LTD.  
24/28 Oval Road, London NW1

Library of Congress Cataloging in Publication Data

Cole, Harold Harrison,      Date      ed.  
Reproduction in domestic animals.

Includes bibliographies and index.

1. Domestic animals—Reproduction. physiology.	I. Cupps, P. T.	2. Veterinary Title.
QP251.C67 1976	596'.01'6	76-9152
ISBN 0-12-179252-8		

PRINTED IN THE UNITED STATES OF AMERICA



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# Preface

The objectives of the third edition of "Reproduction in Domestic Animals" are (1) to outline the basic knowledge relating to the physiological and biochemical and, to a lesser degree, the psychological and pathological aspects of reproduction in domestic animals to produce a text for use by advanced undergraduate students, by graduate students, by research scientists, and by workers in professional fields such as animal science, veterinary medicine, artificial insemination, and livestock consulting; and (2) to review the significant current literature, published since the 2nd edition, so that the book will continue to serve as a significant reference work dealing exclusively with research on reproduction in domestic animals. We assume that those using the text will have an adequate background in the physical and biological sciences.

The initial two-volume edition of "Reproduction in Domestic Animals" with over 1100 pages was the first attempt to collate the literature relating to the physiology of reproduction in domestic animals. The second edition, citing more than 3000 references, was reduced to 657 pages even though four new chapters were added: The Chemistry of the Gonadotropins, The Biochemistry of the Gonadal Hormones, Immunological Characterization of the Gonadotropins, and Oogenesis and Folliculogenesis. To adjust to the spiraling costs of publishing, the third edition has been reduced to less than 700 pages, which includes the index. Most research studies published five or more years previously are referred to through reviews to reduce the space occupied by the reference lists.

We decided to revert to the approach used in the first edition which was to include chapters dealing with special characteristics of the individual domestic species in order to make this information more readily available. A chapter on Reproduction in the Dog and Cat has been added. Because one can best approach the solution of many problems in reproduction on the basis of how hormones act, we have also added a chapter on the Mechanism of Action of Sex Steroid Hormones in the Female. Finally, a chapter appears on Genetic Variation and Improvement.

We were most appreciative of the Foreword by Dr. Herbert McLellan Evans which appeared in the first and second editions of this work. His

death on March 6, 1971, at the age of 89, marked the passing of one of the greatest biologists of modern times. The acme of his career may have been reached in 1921–1922 when he announced: with Dr. Joseph A. Long the discovery of the growth hormone; with Dr. Katherine Scott Bishop the discovery of “a hitherto unrecognized dietary factor essential for reproduction” which later was to be designated vitamin E; and with Dr. Joseph A. Long as the senior author published the historic monograph on the estrous cycle in the rat. A host of significant contributions to our knowledge on the function of the pituitary gland were made in his laboratory, many in collaboration with Dr. Miriam E. Simpson. An excellent bio-bibliography of Dr. Evans prepared by Drs. E. C. Amoroso and G. W. Corner was published in November 1972: *Biographical Memoirs of Fellows of the Royal Society* 18.

We wish to express our gratitude for the scholarly contributions of the authors and for their cooperation in expediting the publication of this edition. The staff of Academic Press has been most helpful. Finally, we are most grateful for the superior assistance of Mrs. Mary Bigelow Horton and Mrs. Magnar (Jane) Ronning in many aspects of the editing.

H. H. Cole  
P. T. Cupps

## Preface to the First Edition

Designing a book to be useful as a text for advanced undergraduate and graduate students, for research workers in the field of reproduction, and for veterinary clinicians presents an interesting challenge. Beyond furnishing an anatomical background, the first six chapters of this book outline modern concepts of reproductive physiology in mammals. The remaining portions deal more specifically with reproduction in domestic animals, the authors, nevertheless, have not hesitated to draw upon knowledge of these events in laboratory animals where they have been elaborately worked out there. Because of its size, this treatise has been divided into two volumes.

Differences in interpretation are not uncommon, the editors have neither desired nor attempted to harmonize viewpoints. These differences reflect the incompleteness of our knowledge. For instance, follicle stimulating and luteinizing hormones, as purified from anterior lobe tissue, have not been clearly demonstrated in the blood or urine of any species. Some authors have assumed that both are true hormones and actually secreted, others have taken a more cautious position.

Usage of terms has, to some extent, been standardized. Consistent usage of the terms "metestrus," "diestrus," and "anestrus" has been difficult to achieve. Inadequate knowledge of the intimate changes in the reproductive organs when Walter Heape introduced the terms accounts in part for this difficulty, species differences in secretory activity of the ovary during the postestrous interval with resulting variations in the complexity of development of the accessory structures is a second complicating factor. Although agreement on the use of these terms would be desirable, the problem is obviously too involved to effect uniformity in the present volumes. Action by appropriate bodies to standardize usage of these terms would be desirable.

The editors take this opportunity to express their appreciation to the authors for preparing their chapters meticulously and promptly. Excellent cooperation by the authors is evidenced in that scarcely more than a year elapsed between receipt of the first manuscript and publication of the book.

For many years reproductive physiology has been greatly enriched by the signal contributions made by Dr. Herbert M. Evans and his colleagues. Many, including the senior editor, have benefited from a sojourn in the inspirational atmosphere of his laboratory. Our thanks are due him as author of the Foreword.

We should like to express our thanks to Miss Lee Doyle and Mrs. June Law for assistance in the preparation of the Subject Index.

Finally, the friendly helpfulness of the Academic Press staff has contributed toward making editing of these volumes a pleasant task.

H. H. Cole  
P. T. Cupps

*Davis, California*  
*March, 1959*

# 1

## Historical Introduction

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### I. Early History

The development of our knowledge of the working of the reproductive processes has been much slower than that of any other of the body functions. The reasons are not difficult to discover. For mammals the non-existence of eggs that could be seen without the aid of the microscope and the time gap between mating and the ability to recognize the products of conception in the uterus were obstacles that could only be surmounted by the invention of suitable visual aids and the formulation of the cell theory. Also, the method of control by hormones, prominent in the functioning of the sexual organs, has been so recent a discovery that the delay is not surprising on this account alone.

Mankind has made up for the lack of physiological knowledge by an abundance of speculation. Probably more has been written and thought about reproduction in one form or another than about anything else. At one time or another every possible suggestion that might account for reproduction of the species or, at any rate, that might explain some facet of the process has been made and pressed with various degrees of plausibility. Most of this speculation and folklore had to be cleared away before explanations acceptable to science could be advanced.

Naturally enough, the Greeks had a multitude of ideas on the subject,



FIG. 1. Edgar Allen, discoverer with E. A. Doisy, of estrogenic activity in liquor folliculi of sow ovaries.



FIG. 2 Left, George W. Corner, discoverer, with Willard M. Allen, of progesterone. Right, Carl G. Hartman, a pioneer in research in primate reproduction.



FIG 3 Philip E Smith (left) and Earl T Engle (right) codiscoverers of the role of the anterior pituitary in reproduction



FIG 4 Herbert M Evans (at right), pioneer in anterior pituitary hormone research and in the relation of vitamins to reproduction. His father and stepmother are with him

and it is now known that many of these ideas had been passed on to them by the wise men of the East, especially India, a country with which they had much contact, particularly at the time of Alexander the Great. Our first comprehensive treatise on reproduction, Aristotle's "Generation of Animals" dates from this period. For a comparable treatise we had to wait over two thousand years until Marshall's "Physiology of Reproduction," appeared. True, Galen (130-200 AD), William Harvey in 1651, discoverer of the circulation of the blood, and others had written books on reproduction, but these were hardly of the quality of the treatises of either Aristotle or Marshall.

The Greek view of reproductive physiology was quite logical if the limited range of the facts at their disposal is considered. The fetus arose from the menstrual blood because this does not appear during pregnancy. This material was activated by the seminal fluid. Sex was determined in this way: the male is darker in color than is the female so, as fetal development depends upon the coagulation of the activated menses, this must, for a male, be conducted at a higher temperature (coagulation of an egg, for instance, requires heat) and this will darken the product. The right side of the body is more active and nobler than the left, so it must be the hotter side. Hence, male fetuses are developed on the right side. Even in present times, one occasionally hears that the right ovary gives rise to males and the left to females. As the two ovaries are said to alternate in their action it is possible, according to this view, to obtain a male or female at will by noting the sex of the previous offspring and counting subsequent cycles. Incidentally, the Greeks of this period had no knowledge of the ovaries. Credit for this discovery is given to the Alexandrine Greek, Herophilus, about a hundred years later (ca. 300 BC). He called them "the female testes," a name which they bore for many centuries. During this interval they were not recognized as equivalent to the ovaries of the bird. Aristotle knew of a bull that remained fertile for a time after it had been castrated and was led to deny a direct influence of the testes upon fertility. He suggested that the semen was derived from all parts of the body and that the testes were merely plummets that kept the necessary tubes from becoming kinked and, thus, stopped up. It is no wonder that these early authorities were confused about the ovaries. This doctrine of semen derivation, known as "pangenesis," was revived in more recent times by Darwin, who called the active particles "gemmules."

After the activities of the Greek Alexandrine School there is little to record until Fallopius, an anatomist at Padua (died 1562, at age 39 years), described the tubules that bear his name. In the intervening centuries there was nothing but practical treatises on obstetrics, and recapitulations of the knowledge brought together by Aristotle, who, with the physi-



cian Galen (130–200 AD), was regarded as the final authority on biological matters. Galen was something of an experimentalist, and he added something to embryology by his dissection of hens' eggs. Then the study of anatomy eventually began to shake off this authoritarianism, and anatomists began to look at specimens for themselves, to dissect them, and to interpret what they saw. A student of Fallopius, Volcherus Coiter, in 1573 described the corpus luteum, and in 1672 Regnier de Graaf described the Graafian follicle. He suggested that it represented the egg, corresponding to the egg of the bird or frog. A few years previously, in 1667, the Danish anatomist Steno had suggested that the female testes were in reality the mammalian equivalent of the ovaries of egg-laying animals, but he had no idea of the nature of the mammalian egg. However, his was a fruitful suggestion and it put the anatomists at last on the right path. De Graaf also killed rabbits at half-hourly intervals after copulation. He discovered that the number of cicatrices in the ovaries usually corresponded with the number of eggs or embryos in the uterus. But he recognized that the "ovum" in the uterus was much smaller than the follicle, which he regarded as the egg.

For many years William Harvey had been studying the subject of reproduction and in 1651, when he was 73 years old, his views were published in book form. He had followed the development of the chick at regular intervals through incubation, as had others before him, and he added materially to our knowledge of embryology. In his mammalian studies he was not so fortunate. He studied the deer and the rabbit, but was baffled by the interval between the time of coitus and the time when something resembling an avian embryo could be found in the uterus. However, he did recognize that the maternal and fetal circulations were distinct, a major contribution in itself.

A few years later, an amateur biologist of Delft, Holland, made an observation that was to mark the beginning of the modern era in this field. He had improved the simple lens and had the curiosity to examine with his instruments everything that came his way. A medical student named Hamm drew his attention to the presence of live animalcules in the semen of a man afflicted with a venereal disease. The biologist van Leeuwenhoek soon found that similar animalcules were present in the semen of males from many species of animals, a fact that was published in print in 1677, though he seems to have informed others by letter several years previously. In those days there was an active correspondence between workers in several countries, and this was an important factor in disseminating the latest information. Publication was secondary. In most countries there were small societies or clubs of philosophical amateurs, who were avid students of the latest scientific discoveries.

This discovery by van Leeuwenhoek led to an immense amount of speculation, and biologists as well as numerous other philosophers keenly debated its implications. They divided into two camps, one maintaining that the ovum, still undiscovered, gave rise to the embryo, while others were equally certain that this was the function of the "new" animalcules. Another controversy arose between the "preformationists" and the "epigenists." The former maintained that the embryo was present fully formed in either the egg or the sperm. Thus, one or other of these bodies must contain the eggs or sperm of the next generation and so on ad infinitum both up and down the line. Adam, or Eve, therefore, must have contained the germs of all their descendants. This absurd doctrine gave rise to much speculation, theological and otherwise, but one gets the impression that several of the writings on the subject may properly be classed in the category of "leg pulls." More sensible were the speculations of Swammerdam (1637-1680), a superb dissector. He was puzzled concerning how the egg got into the oviduct of the frog. He described the external fertilization of the egg and observed its first cleavage. Yet, he was an ardent preformationist. The epigenists took the opposite view, namely, that the egg and the sperm had to be organized into the form of the embryo and that this took place anew with each union of the two. However, there was still only speculation, but no proof, for mammals, that the newly discovered animalcules, or spermatozoa, entered the ovum, and it must be remembered that the actual mammalian ovum had not yet been discovered. Only the early embryo was yet known.

In 1780 the Italian priest Spallanzani, also working with the frog, attempted to answer the question of whether or not the spermatozoa were actually the fertilizing agents for the egg. His method was to filter semen through blotting paper. Fertility was lost in some instances, but not in all. He attempted other experiments of a similar nature, but none gave him the correct answer, as they did not absolutely exclude the spermatozoa. It remained for Dumas in 1825 to provide definite proof by experiments with rabbits that spermatozoa are the fertilizing agents. Spallanzani did show that semen diluted to the extent of one drop in 25 pounds of water retained the ability to fertilize, and he was also the first in modern times to demonstrate the possibility of artificial insemination. He used the dog for this purpose.

Recognition of the mammalian ovum was hardly possible until the cell theory had been developed. Several workers seem to have seen bodies enveloped by the discus proligerus and to have described these as ova. Cruikshank in 1797 may have seen them in this form. He certainly described rabbit ova in the oviducts on the third day following impregnation. Later, Prévost and Dumas opened rabbit follicles and obtained from them bodies about 1 mm in diameter. These were less transparent than the

smaller eggs which they found developing within the uterus. Definite recognition came in 1827 when von Baer described the ovum and noted its relation to the discus proligerus and to the follicle. He called the newly found object an "ovulum." In the same year Dumas said that the spermatozoon and the ovum unite in the oviduct or uterus. He and Prevost saw a single spermatozoon in the egg of a frog and expressed the view that one is sufficient to fertilize the egg. In 1840 Barry found, for the first time, spermatozoa in a mammalian egg. This was a rabbit egg. Three years later he described an embryo in the two-blastomere stage. Ten years after this (1853) Newport described the penetration of a frog's egg by spermatozoa, and the modern age of reproductive biology had begun.

## II. Sex Determination

Sex determination is one aspect of the subject that excited lively controversy, and the theories advanced to account for the sex of an individual, male or female, have been legion. Obviously, no correct answer could be forthcoming until the cell nucleus was recognized and the role of the chromosomes described.

In 1901 McClung pointed out that a sex difference existed in the chromosomes, but his interpretation of it was at fault. It remained for Stevens and Wilson, each working with insects, to work out the true relationship of the heterochromosomes to sex. The haploid nature of the germ cells had been recognized previously in 1883, by van Beneden, and the restoration of the diploid condition by the union of the gametes was already understood. It remained for the recognition of sex linkage by Bateson and Punnett in 1908, a condition that, to clinch the matter, allowed recognition of the line of descent of individual chromosomes. More recent events in this field have been Barr's discovery of the sex chromatin, use of more efficient techniques in chromosome recognition, the finding that the Y chromosome of mammals is definitely concerned in male development, and, most recently, the discovery by Blackler that, in the South African clawed toad, at any rate, the genetic composition of the gonads determines the sex, not the composition of the germ cells themselves. If this latter finding is confirmed in other species, and circumstantial evidence points in this direction, it is an important addition to our knowledge.

## III. Sexual Cycles

One of the first to draw attention to the cyclic nature of the sexual process was Latapie, who worked at Bordeaux with a variety of rodents. This was pioneering work, and at the time attracted little attention. In fact,

the physiology of reproduction was investigated very slowly. After the initial discoveries, the attention of biologists seems to have been diverted in the direction of working out the changes that take place in the cell nucleus during division, and in the study of embryological development. Eventually, both of these led back to reproduction, in the case of embryology because of the demand for more accurately aged material.

Next came the work of Heape, who defined and named the various phases of the female cycle in mammals. He also pointed out the essential similarity between the reproductive processes in man and other mammals, a conclusion he reached as a result of his studies of the changes in the sexual organs of monkeys. Heape also drew attention to the fact that ovulation in the rabbit is induced by coitus. This led to eventual recognition of the endocrine nature of much that goes on in the sexual cycle. He was the first to successfully transfer ova from one female to another.

As a result of Heape's work, Marshall was able to differentiate between the physiological activities of the Graafian follicle and the corpus luteum in their relations to the accessory organs. This work was considerably extended by Ancel and Bouin. Later, Marshall demonstrated the effects of changing light gradients in certain seasonal reproducers, thus helping to explain the nature of this phenomenon.

Further pioneer work was that of Deanesly and Parkes, who showed how, by a carefully contrived sampling system, together with a study of the histology of the tracts, much information may be obtained about reproduction in wild species that do not readily reproduce in captivity.

#### IV. Steroid Hormones

In 1849 Berthold published the results of work in which he castrated roosters and, in a few, implanted the testes upon the intestines. As a consequence, these birds did not display the usual results of castration, and he drew the correct inference that the testes produced blood-borne substances that maintained the accessory sexual structures and male behavior. But it was not until the turn of the century, after Bayliss and Starling had enunciated their hormone theory, that attention was again given to this possibility. In the first decade or so of this century several workers injected various types of crude extracts of gonads or of uteri, and they occasionally obtained growth of the uterus, but, on the whole, the results were not encouraging. In searching for an active substance of totally unknown structure, usually present in very small amounts, a good biological test is essential during the earlier work. In 1917 Stockard and Papanicolaou published their work on the estrous cycle of the guinea pig, and similar

studies followed in 1922, on the mouse, by Allen, and on the rat, by Long and Evans. These studies showed that the vaginal smear gave a valuable clue to the ovarian changes. In 1923, Allen and Doisy used this hint as a means of tracking down the follicular hormone, which was soon isolated. Its structure was worked out, and synthesis soon followed. It turned out to be a steroid substance, a member of a class of organic compounds just beginning to be recognized. At first it was obtained in small amounts and from natural sources only, but when Girard introduced his series of reagents, the isolation in quantity of estradiol and related substances from human pregnancy urine soon followed. A dramatic moment in the history of this development occurred at a meeting called to discuss the possibility of setting up an international reference standard for the estrogens, which had not yet been obtained in an absolutely pure state. The American and British representatives were talking about donating milligrams to the pooled standard when Girard, who had been invited at the last moment, produced from his pocket several grams and promised a good many more.

Isolation of estrogens was quickly followed by that of progesterone from the corpora lutea of pigs by Corner and Allen. The role of this organ in maintaining pregnancy was known from the work in 1903 by Fraenkel, who is said to have received the hint from his professor, Born, while the latter was on his deathbed. The method of detection used by Corner and Allen was to use the extracts to build the endometrium of the ovariectomized rabbit to the level found during pseudopregnancy. The history of these exciting days is recounted in most interesting manner by Corner.

The androgenic activity of testes extracts was discovered by McGee in 1927. He used the sensitivity of the rooster comb in the test. Interest in all these substances was lively, and a number of laboratories were involved in their purification, characterization, and synthesis. Among these may be mentioned the laboratories of Koch, Ruzicka, Butenandt, and Laqueur.

Another hormone, relaxin, has been obtained in impure form from the corpus luteum. It is not a steroid but a polypeptide, and its exact functions are still controversial. It was first described in 1928 by Hisaw.

## V. Anterior Pituitary Hormones

In 1905 Heape suggested that the activity of the gonads was controlled by a substance circulating in the blood. He gave it the provisional name "generative ferment." In 1925 this hypothesis was extended by Hammond and Marshall, who considered that the substance, whatever it

might be, was also concerned in body growth and in lactation, since all these were linked in some way. Growth had to be considerable before the gonads began to function and estrous cycles tended to be suppressed during lactation. If one adds together the functions of several of the anterior pituitary hormones, they act much in the manner suggested by the generative ferment concept.

The first of the anterior hormones to be isolated and purified was prolactin. Evidence for the existence of a pituitary factor that influences milk secretion was first given by Stricker and Grueter in 1928. Riddle and Braucher reported that this hormone stimulated the crop gland of pigeons, a finding which led to a favorite method of assay. It was partially purified and named by Riddle and Bates in 1933. In 1929 Bellerby had shown that an extract of the anterior pituitary gland would induce ovulation in the estrous rabbit, a species that ordinarily requires activation by coitus before ovulation occurs. This was followed by the work of P. E. Smith, who perfected the parapharyngeal surgical technique for removing the rat pituitary, thus providing at the same time evidence regarding pituitary function and a convenient animal for testing the potency of extracts of the gland. The profound effects of the operation upon the gonads, which immediately atrophied, and the restorative effects of implants of the gland or of extracts were soon demonstrated. This work was followed up by Fevold *et al.* and Evans *et al.* and soon led to highly purified preparations of follicle-stimulating hormone (FSH) and luteinizing hormone (LH, or ICSH). Like prolactin both of these were found to be proteins, and their separation proved to be difficult.

As a consequence of relying too much upon evidence from a single species, the rat, which is easier than most to work with, some of the earlier generalizations have not stood up under further testing. For instance, prolactin was found to be the hormone that stimulates the corpus luteum to secrete progesterone. This is so in the rat and probably in many other rodents, but in those species of other orders of mammals about which we have information LH is the activating hormone.

These hormones are evidently used in the glands they stimulate because they have to be supplied continuously if they are to maintain the gonads. Furthermore, there is a relationship between the amount supplied and the amount of their effect. Before they were isolated, Lipschütz had deduced a "law" of follicular constancy. This was based on the fact that females tend to ripen a constant number of follicles at each heat period, and that if one ovary is removed, the other does the work of both. Evidently, the amount of FSH secreted determines the number of follicles that may mature at any one time. Additional injected FSH increases the number of ripening follicles, a fact that has proved useful in experimental work.

Information on the role of the placenta as an endocrine organ came from Aschheim and Zondek's discovery in 1927 of a gonadotropic substance in the urine of pregnant women. It proved to be similar to LH in its physiological properties. This was followed in 1930 by Cole and Hart's discovery of a substance resembling a mixture of FSH and LH in its physiology in the blood serum of the pregnant mare. Both discoveries were useful, as they provided abundant sources for these hormones. It was no longer necessary to rely upon the limited supply of pituitaries for hormones with gonadotropic activity. They were also useful in providing a test for pregnancy in these two species.

In 1937 Harris showed that electrical stimulation of the hypothalamic region of the brain would cause the female rabbit to ovulate, while Markee and his students began to explore the effects of sympathetic and parasympathetic stimulating and blocking drugs upon reproduction. This soon became a very active field, and it is impossible to single out individuals whose contributions have led to the greatest development. It is sufficient to say that the hypothalamus is now recognized as the source of polypeptides that travel to the anterior pituitary and there control the hormone output. It is by this pathway that exogenous stimuli such as light or the sense of smell or sight (Whitten and Bruce effects) are able to exert their effects.

## VI. Physiology of Spermatozoa

By far the most important development in reproductive physiology has been that which has led to the widespread use of artificial insemination in animal breeding. This has obvious advantages in reducing the number of males that must be maintained, in increasing the possibilities of improvement by using only males that transmit to their offspring the most desirable characters, and, especially since the introduction of antibiotics, in controlling the spread of venereal diseases in livestock.

The work of Spallanzani, who successfully inseminated a dog, has been mentioned. After that initial work only sporadic attempts were made to put the discovery into practice until the beginning of this century, when the Russian Iwanow began to investigate the possibility of collecting, preserving, and diluting the semen. His work was, at first, entirely with horses, but he gradually extended it to other domestic species, and after World War I the results began to be used for large-scale purposes. Milovanov was one of those who developed the techniques, and his name is especially associated with the use of semen diluters. In 1936 the first artificial insemination cooperative was organized in Denmark, on the

other deficiencies that may occur during pregnancy. Adverse conditions have to be rather extreme for them to affect reproduction.

A practical problem in which progress has been made is that of synchronization of heats so that a number of females ovulate at a date chosen by the farmer or inseminator. This, when perfected, will make it much easier to arrange a breeding program and will lessen the amount of work and time involved in visiting farms for performing the inseminations. This line of enquiry is still very recent, and it is being pursued in several laboratories. Much of this work consists in screening various steroid derivatives and choosing the most effective ones. These substances are being produced in the research laboratories of many drug houses in connection with the search for the ideal contraceptive. A problem that needs further work is that the conception rate is lower than usual in the heat period immediately after these substances have been used. It is clear that we do not yet have the best means of normalizing the reproductive organs after they have been held inactive by the synchronizing drug.

It is easy to make a list of outstanding problems in reproduction, and a complete one would be very long. Ability to control the sex of the offspring is one that has attracted much attention, and frequent claims of success have been made, only to be followed by a silence that indicates that the method proposed has not been as successful as had at first been believed.

The problem of pineal involvement, which has not been treated in this chapter, is another one that calls for elucidation. Hormone levels and feedback mechanisms also require much more work. The use of radioactive tracers to find the rate of loss of steroid hormones at each step of their assay has already helped in this respect, as it has made assay much more accurate than was possible in the past.

The recent finding of Moss and McCann that LHRH will induce mating behavior ". . . provided that the animals were first primed by the injection of a small quantity of estrogen" shows that much is still to be learned about the physiological effects of the hormones which are known to influence reproductive processes.

There are also many problems of a fundamental nature awaiting answer, such as: What do fertilization and activation of the egg actually involve, and why are they distinct? For that matter, why does a fertilized egg divide? What is the mechanism of reduction division and extrusion of polar bodies? How is ovulation brought about? What causes certain hormones to be so specific in their actions? What are the relationships between the structure of the hormone and that of the cells with which they react? What causes the uteri of some species to be so specific in the positions in which implantation of embryos may occur? And what determines the



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island of Samsø, by Sørensen and Gylling Holm, and from this time progress was rapid.

In the United States an experiment made in Minnesota from 1937 to 1938 proved successful with beef cattle. In 1938 E. J. Perry organized the first American cooperative in New Jersey. Among those who were engaged in developing techniques and in improving the efficiency of operation were Walton, Phillips, and Willett and Salisbury. The latter, in cooperation with Knodt, introduced the addition of sulfa drugs to the semen as a means of reducing bacterial contamination and cutting down the incidence of venereal diseases. About the same time Almquist further improved upon this by adding antibiotics to the semen diluters.

In 1950 Polge *et al.* found that by adding glycerol to bull semen it may be frozen and preserved at  $-79^{\circ}\text{C}$  or lower without undue loss of its fertilizing ability. This discovery, due to a fortunate accident in the laboratory, together with the capacity of the investigators to interpret what had happened, has led to a considerable advance because semen of some species may now be kept for years if necessary; thus, semen banks may be used and specimens sent under refrigeration for very long distances.

Effective use of these new techniques demanded that the estrous cycles, as well as the optimum time in the cycle for insemination and the length of survival both for the sperm and the ovum, should be known in more detail than had previously been the case. Many workers have taken part in these explorations, and it is impossible to mention more than a few of them. In 1903 Marshall published work on the estrous cycle of the sheep, and this was followed by papers by Cole and Miller in 1935 and McKenzie and Terrill in 1937. Murphey was working with cattle in 1924, and Hammond published on the subject in 1927. In 1926 McKenzie dealt with the pig. This was followed in 1959 by a monograph by Nishikawa on the horse.

In 1911 Lewis pointed out that the life of the spermatozoon and of the ovum in the female tract of domestic animals was shorter than was usually realized and gave approximate figures. This was followed in 1926 by a study of the subject in rabbits by Hammond and Asdell which confirmed Lewis's pioneer work. This species was ideal for this type of problem because in it ovulation depends upon coitus, which it follows at a very definite interval. In 1943 Trimmerger and Davis obtained definite figures upon the relationship between estrus and ovulation in the cow and on the effect of the interval between insemination and ovulation upon fertility. Gradually, a satisfactory picture of this very practical phase of the subject has been constructed.

In 1922 Crew suggested that spermatogenesis required a lower temperature than that of the body and that, therefore, the testes were suspended in the scrotum, which serves as a thermoregulator. These facts were used

by Asdell and Salisbury in 1941 to work out the rate of spermatogenesis in the testis. Their results have been essentially confirmed by recent work in which radioactive tracers have given more precise figures.

## VII. Control of the Secretion of Pituitary Hormones

The extension of knowledge during the past decade relating to the control of the secretion of anterior pituitary hormones through hypothalamic hormones and the factors responsible for the release of these hypothalamic hormones undoubtedly represent a major area of advances in our understanding of reproductive physiology. These findings are elegantly described in Chapter 3 and thus need not be considered here.

## VIII. Prostaglandins and Reproduction

There is evidence that prostaglandin  $F_{2\alpha}$  ( $PGF_{2\alpha}$ ) or its precursor, arachidonic acid, is the uterine luteolytic factor in the ewe and cow. (See Chapter 4.) Also it appears that specific uterine proteins may play a significant role in certain processes during pregnancy.

Prostaglandins have been used successfully in the synchronization of estrus in several species (this is alluded to very briefly in Chapter 11). Prostaglandins ( $PGF_{2\alpha}$  or  $PGE_2$ ) have been used successfully in inducing abortion in women (Lobotsky, *Res. Prostaglandins*). Some evidence suggests the prostaglandins may play a role in oviposition in birds; see Chapter 20). One should not be left with the impression that prostaglandins are involved solely in regulating reproductive phenomena. A considerable body of knowledge has been built up to show that "prostaglandins are probably liberated at a large number of autonomic effector sites and that they subsequently influence both the release of transmitter material and the reactivity of the effector organ to the transmitter itself" (M. J. Brody, 1973).

## IX. Miscellaneous Factors

So far nothing has been said about the effects of varying nutrition upon fertility. This is largely because under modern conditions of feeding domestic animals, quantitative and qualitative deficiencies are rarely seen. There is evidence that vitamin A and E deficiencies interfere with spermatogenesis in the rat (Evans and Burr, 1927) but other species do not seem to be as sensitive, and the same seems to be true for vitamin and

other deficiencies that may occur during pregnancy. Adverse conditions have to be rather extreme for them to affect reproduction.

A practical problem in which progress has been made is that of synchronization of heats so that a number of females ovulate at a date chosen by the farmer or inseminator. This, when perfected, will make it much easier to arrange a breeding program and will lessen the amount of work and time involved in visiting farms for performing the inseminations. This line of enquiry is still very recent, and it is being pursued in several laboratories. Much of this work consists in screening various steroid derivatives and choosing the most effective ones. These substances are being produced in the research laboratories of many drug houses in connection with the search for the ideal contraceptive. A problem that needs further work is that the conception rate is lower than usual in the heat period immediately after these substances have been used. It is clear that we do not yet have the best means of normalizing the reproductive organs after they have been held inactive by the synchronizing drug.

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# 2

## Gonadotropins

O. D. Sherwood and W. H. McShan

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### I. Introduction

For nearly 50 years it has been known that gonad stimulating substances exist within the pituitary, blood, urine, and placenta of primates and equidae. Isolation of these gonadotropic hormones proved to be difficult since they are present in small quantities, labile, and, in most cases, polymorphic. New isolation and characterization techniques in the 1960s finally enabled the isolation of several preparations of gonadotropic hormones, which were sufficiently pure to permit their rigorous chemical and physiological characterization.

This chapter will focus attention on chemical and physiological studies which have been conducted with the following gonadotropic hormones: luteinizing hormone (LH), follicle stimulating hormone (FSH), human chorionic gonadotropin (HCG), pregnant mare serum gonadotropin

(PMSG), and prolactin. The volume of literature which has been published within recent years on the chemistry and physiological properties of the gonadotropins is too extensive to review in detail; therefore, we will cite selected examples of recent work. For more extensive coverage of the chemistry of the gonadotropins, the reader is referred to recent reviews (56, 57, 104) and books (75, 106). Recent reviews which describe the physiological characteristics of the gonadotropic hormones have also been prepared (44, 87, 118, 128).

## II. Chemistry of Gonadotropins

### A. COMMON STRUCTURAL FEATURES

#### 1. Carbohydrates

LH, FSH, HCG, and PMSG are glycoproteins. Their carbohydrate contents range from approximately 13% for ovine, porcine, and bovine LH to approximately 45% for PMSG. Generally, within these glycoproteins the monosaccharides—mannose, galactose, fucose, *N*-acetylglucosamine, *N*-acetylgalactosamine, and sialic acid—are linked in multiple oligosaccharide chains to form complex heteropolysaccharide units (6). These complex carbohydrate units are connected to the polypeptide chains with single *N*-acetylglucosaminyl-asparagine linkages. The amino acid triplet sequences, Asn-X-Thr or Asn-X-Ser (X being any amino acid), have been postulated to be the recognition site for the enzyme which attaches *N*-acetylglucosamine to the amide group of asparagine in *N*-glycosidic linkages (117). Little is known about the structures of the carbohydrate units within most gonadotropic hormones; however, Bahl (6) has proposed complete structures for the complex heteropolysaccharide units of HCG and has reported the structure of short oligosaccharide chains linked by *O*-glycosidic bonds between *N*-acetylgalactosamine and serine residues in HCG. The sialic acid in HCG is located terminally on the oligosaccharide chains.

Sialic acid is essential for full expression of the biological activity of several gonadotropins. Complete enzymic removal of sialic acid from human LH (18), HCG (122), ovine, porcine, and chicken FSH (68), and PMSG (127) drastically reduced their biological activity. Morell *et al.* (79) have proposed that sialic acid prolongs the half-life of glycoproteins by inhibiting their uptake and degradation by the liver. Sialic acid apparently contributes little or nothing to the structural configuration required for interaction of glycoprotein hormones with their target tissue receptors.

Following the complete removal of sialic acid from HCG, the plasma half-life was reduced more than 95%, whereas its affinity for ovarian binding sites was not diminished (121, 123).

## 2. Subunits

The gonadotropins LH, FSH, HCG, and PMSG, as well as thyroid-stimulating hormone, consist of two dissimilar subunits designated  $\alpha$  and  $\beta$  which can be readily dissociated by incubation with urea, guanidine-HCl, or propionic acid. These subunits possess little (104) or no (45, 62) biological activity. The lack of biological activity appears to be due to a lack of affinity of the subunits for hormone receptors within tissues responsive to the intact molecule. For example, highly purified  $\alpha$  and  $\beta$  subunits of HCG fail to inhibit the binding of  $^{125}\text{I}$ -labeled HCG to homogenized testis (22) preparations. The recombination of glycoprotein hormone subunits generally results in the regeneration of from 50 to 80% of the original biological activity (45, 62, 104). Chemical and biological studies have led to the concept of a common  $\alpha$  subunit and hormone-specific  $\beta$  subunit. Figure 1 shows that the amino acid sequences of the  $\alpha$  subunits of LH, HCG and FSH are similar but not identical to one another. In contrast, Fig. 2 shows that there is considerable variation in amino acid sequences and/or lengths of the  $\beta$  subunits among the three hormones LH, HCG, and FSH. The  $\beta$  subunit has the structural characteristics which are required for a specific hormone activity. Therefore, hybrid combinations of the  $\alpha$  subunit of one hormone with the  $\beta$  subunit of another hormone generally results in the recovery of considerable biological activity of the hormone from which the  $\beta$  subunit was obtained. For example, a combination of the  $\alpha$  subunit of LH with the  $\beta$  subunit of FSH yields a hybrid with FSH activity (57).

## 3. Polymorphism

Jutisz and Tertrin-Clary (57) and Sairam and Papkoff (104) give a detailed discussion of gonadotropic hormone polymorphism.

Studies with highly purified ovine LH (110), bovine LH (29), porcine LH (1), equine LH (17), human LH (94), ovine FSH (111), HCG (41), and PMSG (39) indicate that these glycoprotein hormone preparations consist of a spectrum of physically and chemically similar molecules which differ from one another to a degree required for their chromatographic and/or electrophoretic resolution.

The polymorphism of LH has been attributed to microheterogeneity of the carbohydrate units (72), deletion of amino acids from the N- and C-



termini (69, 71), genetic differences in amino acid sequences (71), and differences in amide content (37). The microheterogeneity of HCG has been generally attributed to differences in sialic acid content (57).

The polymorphism of glycoprotein gonadotropins may be the result of natural *in vivo* structural variations among molecules. Alternatively, enzymic and chemical alterations may occur in the native molecule during isolation or storage. Both of these explanations may contribute to this polymorphism. Withdrawal of physiological amounts of gonadal steroids by gonadectomy changes the apparent molecular size of pituitary FSH in the monkey (95) and rat (13). These observations (13, 95) support the point of view that gonadotropic hormone polymorphism might, in part, be a natural physiological phenomenon.

## B. LUTEINIZING HORMONE (LH)

Highly purified preparations of ovine, bovine, porcine, equine, and human LH have been prepared in sufficient yields to permit rigorous characterization of their chemical properties. Avian pituitaries also contain two glycoprotein gonadotropins. Gonadotropic hormone preparations with chemical and biological properties similar to mammalian LH and FSH have been isolated from chicken (119) and turkey pituitaries (36). These avian gonadotropins demonstrate relatively low biological potency when assayed in the rat; therefore, they are generally assayed in more responsive nonmammalian systems (36). Chemical studies of the avian gonadotropins are in their infancy.

### 1. Yields and Biological Activity

Table I summarizes the yields, biological activities, and physical properties of several preparations of mammalian gonadotropins. For more information concerning the isolation techniques which have been employed, the reader is referred to the recent reviews by Jutisz and Tertrin-Clary (57) and Sairam and Papkoff (104).

The wide variation in yields of gonadotropin preparations may be attributed to a number of factors including differences in the purity of preparations and differences in the quantity of biologically active molecules present in the original extract which were retained throughout isolation. With most mammalian species the yields of LH are relatively high, generally exceeding 100 mg/kg whole frozen pituitary tissue. Equine LH is an apparent exception since the yield of the highly purified preparation of Braselton and McShan (17) is only 16 mg/kg whole frozen pituitary tissue.

TABLE I

Characteristics of LH, FSH, HCG, and PMSG

Hormone and species	Yields (mg/kg) frozen whole pituitary tissue	Relative potency	Physical properties		References
			Molecular weight by sedimentation analysis	Isoelectric point by isoelectric focusing	
LH <sup>a</sup>					
Ovine	30-380	1.9-4.5	28,000-32,500	7.0-9.4	57, 104, 110
Bovine	60-180	1.5-2.1	25,200-30,000	9.55	29, 57, 104
Porcine	270	0.7-3.9	27,000-34,000	7.4-7.8, 9.80	1, 57, 104
Equine	16	5.3-6.2	33,500	4.5-7.3	17, 61
Human		8.0-10.7	34,000	6.76-9.85	94, 100, 120
FSH <sup>b</sup>					
Ovine	2.7	133	32,700-33,800	4.6	111
Bovine		3.1		4.82	98, 100
Equine	26	90	33,200	4.1	17
Human	10	390-428	32,600	3.36-5.55	93, 100, 101, 105
HCG <sup>c</sup>					
Human		12,000-18,500	37,700	3.8-5.1	41, 81
PMSG <sup>d</sup>					
Equine		15,000-16,000	28,000		15, 64

<sup>a</sup> Relative potencies determined by ovarian ascorbic acid depletion bioassays and expressed relative to NIH LH-S1

<sup>b</sup> Relative potencies determined by ovarian HCG augmentation bioassays and expressed relative to NIH FSH-S1

<sup>c</sup> Relative potencies determined by rat ventral prostate weight bioassays and expressed relative to 2nd IRP HCG

<sup>d</sup> Relative potencies determined by rat ovarian weight bioassay and expressed relative to 2nd international standard PMSG

Relative potencies of LH preparations are generally determined with the rat ovarian ascorbic acid depletion bioassay and expressed in terms of the partially purified ovine LH preparation NIH-LH-S1 (see Chapter 5).

Although relative potencies of LH preparations obtained from several species overlap, rigorous biological characterization of highly purified preparations of LH indicate that species differences in the biological properties of LH probably exist. Highly purified ovine and bovine LH preparations are generally two to three times as active as the NIH-LH-S1 standard, whereas porcine LH preparations are, in nearly all cases, approximately equal to the NIH-LH-S1 standard. The highly purified equine LH preparation of Braselton and McShan is 5.3 (17) to 6.2 (61) times as active as

NIH-LH-S1 and is apparently unique among LH preparations in that it also contains an FSH component with a different electrophoretic mobility than the major equine FSH fraction.

Human LH preparations demonstrate the highest LH activity. Preparations 8.0 to 10.7 times more active than the NIH-LH-S1 standard have been reported (94, 97, 120).

## 2. Physical Properties

Molecular weight and isoelectric points of LH obtained from different species are shown in Table I. The molecular weights are approximately 30,000 for all species with the possible exception of equine and human LH which may be slightly larger. LH preparations are polymorphic (Section II,A,3) and generally demonstrate a broad range in isoelectric points. Table I shows that equine LH molecules which contain a relatively high content of sialic acid have a lower range of isoelectric points than ovine, bovine, and porcine LH which lack sialic acid.

## 3. Carbohydrate Moiety

The relatively high carbohydrate composition of equine LH (Table II) is primarily attributable to sialic acid which is absent or found in low quantities in the LH of the other species. The high biological activity of equine LH may in part be attributable to its high sialic acid content. The plasma half-life of equine LH was reported to be 270 minutes following its administration into rats, whereas the plasma half-life of ovine LH was only 15 minutes (92). The carbohydrates of ovine, bovine, porcine, and human LH have been reported to be found in two heteropolysaccharide chains linked to the  $\alpha$  subunit and one heteropolysaccharide chain linked to the  $\beta$  subunit (Figs. 1 and 2).

## 4. Primary Structure

The LH- $\alpha$  subunits of ovine, porcine, and human LH contain 89-96 amino acids (Fig. 1). The amino acid sequences of these three hormones are similar but not identical. Porcine LH- $\alpha$  lacks six amino acids found on the N-terminus of ovine LH- $\alpha$  and contains three additional amino acid substitutions. Human LH- $\alpha$  lacks seven amino acids found on the N-terminus of ovine LH- $\alpha$  and contains 24 additional amino acid substitutions.

Figure 2 shows the close agreement in amino acid sequences of the LH- $\beta$  subunits of four species. The LH- $\beta$  subunits of ovine, bovine, and porcine LH contain 119 amino acid residues. The LH- $\beta$  subunit of human LH

TABLE II

Carbohydrate Composition of LH, FSH, HCG, and PMSG

Hormone and species	Carbo- hydrate (%)	Carbohydrate composition in residues per mole						NANA Ref.
		Man <sup>a</sup>	Gal	Fuc	GluNAc	GalNAc		
LH								
Ovine	13.0	7.2 <sup>b</sup>	1.1	1.6	8.5	3.4	0	57
Bovine	12.2	9.0	0	1.6	0	10.0	0	57
Porcine	13.2	8.4	1.2	1.5	8.1	3.0	0	57
Equine	23.6	8.1	6.2	1.2	9.0	5.5	8.5	61
Human	16.4	8.3	3.7	2.0	11.3	1.9	2.3	57
FSH								
Ovine	23.9	9.9	5.1	1.6	14.2	2.4	6.3	45
Equine	24.2	10.7	5.6	2.1	10.0	4.4	6.8	62
Human	25.9	21.4 <sup>c</sup>			16.9 <sup>d</sup>		5.6	99
HCG	29.0-30.3	11.4	12.1	1.5	16.4	3.5	9.5-10.9	82
PMSG <sup>e</sup>	44.4	6.2	20.2	2.4	18.3	3.6	9.4	16

<sup>a</sup> Abbreviations are as follows: mannose, Man, galactose, Gal, fucose, Fuc, *N*-acetylglucosamine, GluNAc, *N*-acetylgalactosamine, GalNAc, sialic acid, NANA.

<sup>b</sup> Residues per mole assuming the following molecular weights: ovine, bovine, and porcine LH, 30,000 (57); equine LH, 34,000 (61); human LH, 34,000 (120); ovine FSH, 33,000 (111); equine FSH, 33,800 (17); human FSH, 32,600 (105); HCG, 37,700 (81); PMSG, 28,000 (15).

<sup>c</sup> Total hexose.

<sup>d</sup> Total hexosamine.

<sup>e</sup> PMSG also reported to contain 2.5 residues of glucose per mole (16).

contains slightly fewer amino acid residues. The amino acid sequence of ovine LH- $\beta$  reported by Liu *et al.* (70) does not differ from the sequence of bovine LH- $\beta$  reported by Maghuin-Rogister and Hennen (73). The sequence of porcine LH- $\beta$  (73) differs from ovine LH- $\beta$  by only 15 amino acid substitutions. The human LH- $\beta$  structure proposed by Shome and Parlow (113) contains 115 rather than 119 amino acid residues, and the single heteropolysaccharide unit is linked to the asparagine in the 30 position rather than in the 13 position as with the other LH- $\beta$  subunits. Thirty-seven residues of human LH- $\beta$  differ from those in identical positions within ovine LH- $\beta$ . The positions of the disulfide bonds and amido groups within both the  $\alpha$  and  $\beta$  subunits of LH remain uncertain.

### C. FOLLICLE-STIMULATING HORMONE (FSH)

Chemical and physiological studies of FSH lag behind those of LH and HCG primarily because of the difficulties encountered in isolating FSH

	5	10	15	
o LH	H Pro-Asn Gly-Glx-Phe-Thr-Met-Gln-Gly-Cys-Pro-Glx-Cys-Lys-Leu-Lys Glx-Asx-Lys-Tyr-Phe-Ser-Lys Pro-Asx-Ala-Pro-Ile-Tyr-			
p LH	H-Thr-			-Leu-Gly-
h LH	H-Val-	-Asp-	-Thr-	-Gln-
h CG	H Ala Pro-Asx-Val-	-Asx-	-Thr-	-Glx-
h FSH	H-Val-	-Asp-	-Thr-	-Glx-
				55 CHO
	35	40	45	50
o LH	Glx-Cys Met Gly-Cys-Cys-Phe-Ser-Arg-Ala-Tyr-Pro-Thr-Pro-Ala-Arg-Ser-Lys-Lys-Thr-Met-Leu-Val-Pro Lys-Asn-Ile-Thr-Ser-Glx-			
p LH				
h LH			-Leu-	-Gln-
h CG			-Leu-	-Gln-
h FSH			-Leu-	-Gln-
				85 [Ser-Cys]90
	65	70	75	80
o LH	Ala-Thr-Cys-Cys-Val-Ala Lys-Ala Phe-Thr Lys Ala-Thr-Val-Met Gly-Asn-Val-Arg-Val Glx-Asn-His-Thr-Glx-Cys His-Cys-Ser-Thr-			
p LH				Ala-
h LH		Ser-Tyr Asn-Arg-Val-		-Gly-Phe-Lys-
h CG	Ser-	Ser-Tyr Asn-Arg-Val-		-Gly Phe-Lys-
h FSH	Ser-	Ser-Tyr-Asn Arg Val		-Gly-Phe-Lys-
				Ala-
				Ala-
				Ala-
				Ser-Cys-
	95			
o LH	Cys-Tyr-Tyr-His Lys-Ser OH			
p LH	Ser-OH			
h LH	Ser-OH			
h CG	Ser-OH			
h FSH	Ser-OH			

FIG. 1. Comparison of the amino acid sequences of porcine LH- $\alpha$ , human LH- $\alpha$ , HCG- $\alpha$ , and human FSH- $\alpha$  with ovine LH- $\alpha$ . Residues which are not identical to ovine LH- $\alpha$  are shown. The amino acid sequence of ovine LH- $\alpha$ , shown in this figure, was determined by Liu *et al.* (69). The amino acid sequence of ovine LH- $\alpha$  reported by Sairam *et al.* (102) differs from that of Liu *et al.* (69) by only the two amino acids which are enclosed in brackets at residues 88 and 89. The sequence of porcine LH- $\alpha$  was determined by Maguin-Rogister *et al.* (74) and that of human LH- $\alpha$  by Sairam *et al.* (103). The sequence of HCG- $\alpha$  was determined by Bellisario *et al.* (11). The sequence of FSH- $\alpha$  was determined by Shome and Parlow (114).

and the relatively low yields of this hormone which are obtained. Preparations of FSH sufficiently pure for rigorous characterization have been reported only from sheep (111), horse (17), and human (93, 101) pituitaries. Employing the procedure of Sherwood *et al.* (111), H. J. Grimek and B. C. Wentworth have recently isolated bovine FSH which is approximately 30 times as active as NIH-FSH-S1 (personal communication).

### 1. Yields and Biological Activity

Table I shows that the yields of highly purified pituitary FSH vary among species and tend to be lower than the yields of pituitary LH. The relative potency of FSH preparations is generally determined with the rat ovarian HCG augmentation bioassay and expressed in terms of the very impure ovine FSH preparation NIH-FSH-S1. Available evidence indicates the ovine FSH preparation of Sherwood *et al.* (111), reported to be 133 times as active as NIH-FSH-S1, and the equine FSH preparation of Braselton and McShan (17), reported to be 90 times as active as NIH-FSH-S1, are homogeneous. The human FSH preparations of Roos and Gemzell (101) and Peckham and Parlow (93) are approximately 400 times as active as NIH-FSH-S1 and are the most active FSH preparations reported. The differences in the abilities of these apparently homogeneous FSH preparations to stimulate ovarian growth in the rat makes it seem probable that there are variations in FSH molecular structure among different species.

### 2. Physical Properties

The molecular weights of ovine, equine, and human FSH are approximately 32,000 (Table I). With the exception of the horse, isoelectric points of FSH are considerably lower than those of LH from the same species (Table I). The relatively acid nature of FSH is probably due to the presence of sialic acid which is not found in ovine or bovine LH and is present only in small amounts in human LH (Table II). FSH does not generally demonstrate the broad electrophoretic polymorphism frequently seen with LH and HCG as demonstrated by the relatively narrow range of isoelectric points (Table I).

### 3. Carbohydrate Moiety

Carbohydrates comprise about 25% of ovine, equine, and human FSH (Table II). Sialic acid, not found in ovine LH, is present in ovine FSH;



	95	100	105	110	115	120
o/b LH	Gly Pro-Cys Arg Leu Ser Ser Thr Asp-Cys Gly Pro Gly Arg Thr Glx Pro Leu Ala-Cys Asx His Pro Pro Leu Pro Asp Ile Leu OH					
p LH		Ser	Ala			Gly Leu Leu-OH
h LH	Arg	Thr Ser	Gly Pro Lys Asx His	Thr	Glx His (Ser Lys Gly)-OH	
h CG	Ala Leu	Thr Thr	Gly Pro Lys Asp His	Thr	Asp Arg Phe Glu	CHO Ser-Ser-Ser
h FSH	Cys Asp-Ser Asp-Ser Thr Asp-Cys Thr Val Arg Gly Leu Gly Pro Ser Tyr-Cys Ser Phe Gly Glu Met (Glx, Lys)					
	125	CHO	CHO	135	140	145
						Pro Ile Leu Pro Glu-Ser Leu Pro-OH

CG Lys Ala Pro Pro Ser Leu Pro Ser Pro Ser Arg Leu Pro Gly Pro Pro Asx

FIG. 2. Comparison of the amino acid sequences of porcine LH- $\beta$ , human LH- $\beta$ , and human FSH- $\beta$  with ovine and bovine LH- $\beta$ . Residues which are not identical to ovine and bovine LH- $\beta$  are shown. Two gaps, designated with an X, were placed in the amino acid sequence of human LH- $\beta$  in order to obtain the most nearly identical agreement with the sequences of the other  $\beta$  subunits shown. The sequence of ovine LH- $\beta$  was determined by Liu *et al.* (70) and that of bovine LH- $\beta$  and porcine LH- $\beta$  by Maghain Rogister and Hennin (73). The sequence of human LH- $\beta$  shown in the figure was proposed by Shome and Parlow (113). The amino acid sequence of human LH- $\beta$  proposed by Colset *et al.* (26), which is not shown in this figure, differs slightly from that proposed by Shome and Parlow (113). However, the structure of Closset *et al.* (26) contains an additional heteropolysaccharide unit at position 13. The sequence of HCG  $\beta$  is that reported by Carlsen *et al.* (20) and that of FSH  $\beta$  by Shome and Parlow (115).



and its enzymic removal renders ovine FSH inactive when tested by *in vivo* bioassays (68). The carbohydrates of human FSH have been reported to be found in four heteropolysaccharide units, two linked to the  $\alpha$  subunit and two linked to the  $\beta$  subunit (Figs. 1 and 2).

#### 4. Primary Structure

The primary structures of human FSH- $\alpha$  (114) and human FSH- $\beta$  (115) have been reported. The  $\alpha$  subunit of human FSH contains 89 amino acids. The amino acid sequence of human FSH- $\alpha$  does not differ from that of human LH- $\alpha$  (Fig. 1). The amino acid sequence of human FSH- $\alpha$  differs from that of HCG- $\alpha$  by only three additional amino acids at the N-terminus of HCG- $\alpha$ . The FSH- $\beta$  subunit contains 115 amino acids (Fig. 2). The amino acid sequence of the hormone specific  $\beta$  subunit of human FSH differs markedly from the  $\beta$  subunit of human LH. Human FSH- $\beta$  contains only seven amino acids which are identical to those in the same positions within human LH- $\beta$ . In addition, human FSH- $\beta$  contains two heteropolysaccharide units at positions 7 and 24 rather than one at position 13.

#### D. HUMAN CHORIONIC GONADOTROPIN (HCG)

Human chorionic gonadotropin (HCG) was discovered in human pregnancy urine by Aschheim and Zondek (4) in 1927. The site of synthesis of HCG is the syncytiotrophoblastic cells of the placenta. Levels of HCG in the urine rise to a maximum during the first 10 to 12 weeks of pregnancy and decline to a lower level during the last one-half of pregnancy.

##### 1. Biological Activity

The biological properties of HCG are somewhat similar to those of LH; therefore, the rat ovarian ascorbic acid depletion and rat ventral prostate weight bioassays, commonly used to assay LH, are also used to determine the biological activity of HCG preparations. Biological activity is usually expressed relative to the 2nd international standard for HCG (2nd IRP-HCG) which contains one international unit (IU) per 0.001279 mg (57).

Several laboratories (57) have employed commercial preparations of HCG containing 1500–3000 IU/mg as starting material from which

highly purified preparations of HCG containing 12,000–18,000 IU/mg have been obtained

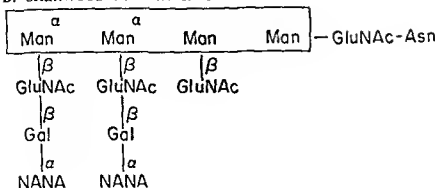
## 2 Physical Properties

Human chorionic gonadotropin is somewhat larger than human LH and human FSH (Table I). A few sedimentation equilibrium analyses have indicated that the molecular weight of HCG is 40,000 or more (57). However, Mori (81), employing sedimentation equilibrium, obtained a molecular weight of 37,700, which is in excellent agreement with the molecular weight of 37,900 determined on the basis of the chemical composition of HCG (11, 20).

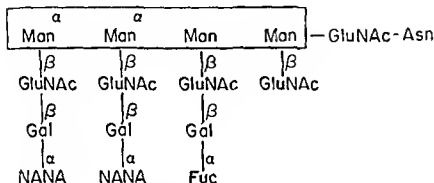
The isoelectric point range of HCG shown in Table I is lower than that of human LH, primarily because of its relatively high sialic acid content (Table II). The microheterogeneity of HCG appears to be attributable to variations in sialic acid content among HCG molecules within highly purified HCG preparations (57). Graesslin *et al* (41) employed isoelectric focusing to fractionate a preparation of highly purified HCG into five biologically active components. The fraction with the highest sialic acid content had the lowest isoelectric point at pH 3.8, and the fraction with the lowest sialic acid content had the highest isoelectric point at pH 5.1.

## 3 Carbohydrate Moiety

Bahl (6) has proposed that human chorionic gonadotropin contains seven carbohydrate units which collectively comprise approximately 30% of the weight of the molecule. Four of the heteropolysaccharide units consist of 10 to 15 monosaccharide residues. These complex units are linked to the polypeptide chain by *N*-acetylglucosaminyl-asparagine linkages as described in Section II A.1. Bahl (6) has proposed the tentative structures I and II (Fig. 3) for the monosaccharide sequences of these four complex carbohydrate units. According to Bahl, both of the carbohydrate units in HCG- $\alpha$  (Fig. 1) have structure I, and the asparagine-linked carbohydrate units in positions 13 and 30 of HCG- $\beta$  (Fig. 2) have structure II. The three remaining small carbohydrate units are composed of NANA-Gal-GalNAc and are linked by *N*-acetylgalactosaminyl-serine linkages at positions 118, 129, and 131 of HCG- $\beta$  (Fig. 2).



Structure I



Structure II

FIG. 3. Tentative structures suggested for the heteropolysaccharides of HCG (6).

#### 4. Primary Structure

The amino acid sequence of HCG- $\alpha$  proposed by Morgan *et al.* (80) is in agreement with the sequence proposed by Bellisario *et al.* (11) (Fig. 1). The  $\alpha$  subunit of HCG contains 92 amino acids and is identical to human LH- $\alpha$  with the exception of three additional amino acids on the N-terminus. The amino acid sequence of HCG- $\alpha$  is also similar to those of ovine and porcine LH- $\alpha$ . Compared to ovine LH- $\alpha$ , HCG- $\alpha$  contains 25 amino acid replacements. The two carbohydrate units within HCG- $\alpha$  are linked to asparagine residues homologous to those linking carbohydrate within ovine, porcine, and human LH- $\alpha$  and FSH- $\alpha$ . Figure 2 shows that the amino acid sequence of HCG- $\beta$  proposed by Carlsen *et al.* (20) is similar to that of ovine LH- $\beta$ , porcine LH- $\beta$ , and human LH- $\beta$  but dissimilar to that of human FSH- $\beta$ . The  $\beta$  subunit of HCG contains two structural features which are not found with LH- $\beta$  or FSH- $\beta$ . There are 28 additional amino acid residues on the C-terminus of HCG- $\beta$  which are not present on ovine LH- $\beta$  or the  $\beta$  subunits of the other gonadotropins. There are three additional carbohydrate units on HCG- $\beta$  which are linked to serine residues located at positions 118, 129, and 131. Morgan *et al.*

(80) have proposed a primary structure of HCG- $\beta$  which is similar to that of Carlsen *et al.* (20). Perhaps the most noteworthy difference is the presence of four rather than three carbohydrate units linked to serine residues near the C-terminus of the subunit.

## E. PREGNANT MARE SERUM GONADOTROPIN (PMSG)

The discovery, levels of PMSG in the blood, and the source of this hormone are discussed in Chapter 15; the morphology of the endometrial cups is considered in Chapter 12.

### 1. Biological Activity

The most frequently used bioassay for PMSG is the rat ovarian weight bioassay described in Chapter 5. Biological activity is usually expressed relative to the 2nd international standard for PMSG which contains 1 IU per 0.003569 mg (7). Since the late 1950's several laboratories have reported techniques for the isolation of PMSG preparations containing high biological activity (56). Recently, Gospodarowicz (39) and Schams and Papkoff (107) have reported relatively simple techniques for the preparation of PMSG with high biological potency.

### 2. Physical Properties

The molecular weight of PMSG remains uncertain. Bourrillon and Got (15) obtained a molecular weight of 28,000 for PMSG employing sedimentation analysis. However, size determinations by means of sodium dodecyl sulfate polyacrylamide gel electrophoresis (39) suggest PMSG may have a molecular weight as high as 53,000. Papkoff (90) recently reported the dissociation of PMSG into  $\alpha$  and  $\beta$  subunits which possessed very low biological activity. Recombination of these subunits restored both the FSH-like and LH-like biological properties of PMSG. Bourrillon and Got (14) employed zone electrophoresis to obtain an isoelectric point of 1.8 for PMSG.

### 3. Carbohydrate Moiety

Table II shows the carbohydrate composition of PMSG as reported by Bourrillon *et al.* (16). The carbohydrate content of PMSG is higher than that of the other gonadotropins, comprising nearly 50% of the weight of the molecule. The sialic acid content of PMSG is high (16, 39) and small quantities of glucose have been reported (56).

## F. PROLACTIN

### 1. Yields

Preparations of prolactin sufficiently pure for characterization studies have been obtained from ovine (28), porcine (34), bovine (54), and human (53) pituitaries. The yields of human prolactin are approximately 100 mg/kg frozen pituitary tissue (53), whereas the yields of ovine prolactin are as high as 4 gm/kg frozen pituitary tissue (67).

### 2. Physical Properties

Ovine, bovine, porcine, and human prolactin have molecular weights of approximately 22,500. From the structure of ovine prolactin, the isoelectric point was calculated to be pH 5.1 (67) and the isoelectric point of porcine prolactin was experimentally determined to be pH 4.9 (34).

### 3. Primary Structure

The primary structures of ovine, bovine, and porcine prolactin are shown in Fig. 4. Li (66, 67) has reported that ovine and porcine prolactin consist of a single chain which contains 198 amino acid residues and three intrachain disulfide linkages located between residues 4-11, 58-173, and 190-198. As compared to ovine prolactin, there are 36 amino acid replacements in the amino acid sequence of porcine prolactin. The primary structure of bovine prolactin proposed by Wallis (124) differs only slightly from that proposed for ovine prolactin. Bovine prolactin contains an additional leucine at position 88 and two amino acid replacements at positions comparable to residues 107 and 164 of ovine prolactin. Niall *et al.* (86) have determined the sequence of the first 40 N-terminal amino acids of human prolactin; as compared to ovine prolactin, there are only 9 amino acid substitutions.

## III. Physiological Characteristics of Gonadotropins

### A. GENERAL REMARKS

Several advances on biochemical aspects of gonadotropic hormone research have accelerated the progress of studies on the physiological characteristics of the gonadotropins. Two advances which appear particularly noteworthy are the following: (a) Highly purified preparations of gonadotropic hormones which have met rigorous biological and physico-

[illegible]

Fig. 4 Comparison of the amino acid sequences of bovine and porcine prolactin with ovine prolactin. Residues which are not identical to ovine prolactin are shown. The primary structure of ovine prolactin was proposed by Li *et al.* (65). The sequence of bovine prolactin was determined by Wallis (124) and that of porcine prolactin by Li (66).

chemical criteria of purity are now available. The interpretation of physiological studies with these gonadotropins need not be qualified on the basis of possible significant contamination. (b) Specific and sensitive radioimmunoassays for the gonadotropic hormones and the gonadal steroids have been developed so that the serum levels of these hormones can now be readily measured under a variety of physiological conditions.

The pituitary gonadotropins LH and FSH differ physiologically as well as chemically. The physiological properties which are unique to each of these gonadotropins form the basis of the specific bioassays which are used to determine the degree of purity or level of cross-contamination of LH and FSH preparations. In spite of these differences in their biological properties, there are some physiological events such as luteinization, ovulation, and steroidogenesis which can be initiated by either LH or FSH. Therefore, caution must be exercised in the assignment of specific physiological characteristics to LH and FSH. It appears probable that under physiological conditions both LH and FSH are utilized simultaneously or sequentially to obtain a given response. When considering the *in vivo* physiological characteristics of the gonadotropins, attention should also be given to the widely differing plasma half-lives of these hormones. In the rat, the plasma half-life of biologically active endogenous LH averaged 30 minutes, whereas that of endogenous FSH averaged 149 minutes (12). The plasma half-lives of HCG and PMSG after exogenous administration into rats were determined to be 4.9 and 26 hours, respectively (91). The relatively long plasma half-lives of HCG and PMSG are thought to be attributable to their relatively high sialic acid contents (Table II).

The pattern of gonadotropic hormones secreted throughout the estrous cycle in mammals is in general agreement with that of the ewe (Fig. 5). It is characterized by a sharp rise and fall in the levels of both LH and FSH just before ovulation. The release of this so-called "preovulatory surge" of both LH and FSH from the anterior pituitary gland appears to be brought about by a decapeptide which is produced in the hypothalamus. Schally and his co-workers (5) determined the structure of this LH- and FSH-releasing hormone (LH-RH and FSH-RH) to be (pyro)Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly(NH<sub>2</sub>).

## B. LUTEINIZING HORMONE (LH) OR INTERSTITIAL CELL STIMULATING HORMONE (ICSH)

### 1. Physiology of LH in the Female

The reader is referred to Greenwald (44) for a more extensive review of the role of LH and FSH in follicular development and ovulation. The role

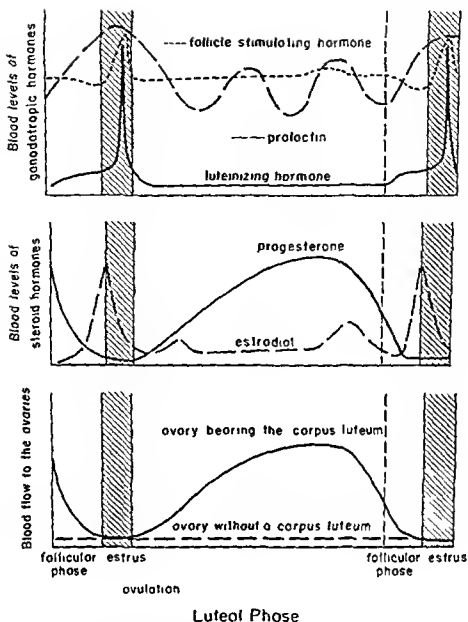


FIG. 5. Blood levels of gonadotropic and steroid hormones and ovarian blood flow throughout the estrous cycle of the ewe (88).

of LH in the transformation of a growing follicle to a fully developed vesicular follicle under normal physiological conditions remains uncertain. Administration of highly purified preparations of LH into hypophysectomized female rats stimulates the development of interstitial tissue without bringing about marked growth of vesicular follicles (27, 109, 110). It has been suggested that LH may be necessary to complement the actions of FSH in the development of vesicular follicles (44). However, the administration of low doses of highly purified ovine FSH, apparently devoid of LH, caused the development of vesicular follicles in hypophysectomized female rats (109).

Both the LH and FSH present in the preovulatory surge of gonadotropins, shown in Fig. 5, probably play a role in the initiation of ovulation.



Nuti *et al.* (89) induced ovulation in ovine FSH-primed immature hypophysectomized female rats with either highly purified ovine FSH or highly purified ovine LH. LH may be the principle ovulatory hormone. The induction of ovulation in ovine FSH-primed immature hypophysectomized female rats required 1.28  $\mu$ g of highly purified ovine LH, whereas 8  $\mu$ g of highly purified ovine FSH were required (89). Schwartz *et al.* (108) blocked ovulation in rats with antisera to ovine LH, whereas antisera to ovine FSH did not block ovulation. There is considerable evidence, however, that FSH may also play a role in ovulation. Goldman and Mahesh (38) blocked ovulation in the hamster by administering a crude antiserum to LH shortly before the anticipated time of ovulation. The removal of FSH antibodies from this crude LH antiserum reduced its ability to block ovulation, thereby indicating that FSH may also be involved in ovulation. Jones and Nalbandov (55) administered ovine LH (NIH-LH-S15) or ovine FSH (NIH-FSH-S6) or combinations of these gonadotropins by both systemic injections or direct injections into the lumen of antral follicles of rabbits. With both techniques they found that LH and FSH individually caused ovulation. However, when combinations of LH and FSH were injected, synergism of the two hormones appeared to occur. The total doses of the combined hormones required to obtain the maximal ovulation rate were much lower than those required for either hormone when they were administered individually. Nalbandov and Bahr (84) have concluded that although LH or FSH can individually "force" ovulation, under physiological conditions ovulation occurs most readily when both hormones are present. They have suggested that the preovulatory surge of LH and FSH should be regarded as an "ovulation-inducing hormone" complex.

For a more comprehensive discussion of the role of gonadotropins in the initiation and maintenance of luteal function, the reader is referred to the reviews by Nalbandov (83) and Nicoll (87). Both LH and FSH probably play a role in the initiation of luteinization. Evidence supporting the role of FSH is described later in this chapter (Section III,C,1). Evidence supporting the role of LH in the initiation of luteinization is as follows. When granulosa cells of everted rabbit Graafian follicles were incubated with ovine LH (NIH-LH-S14) and then autotransplanted beneath the kidney capsule, they developed into corpora lutea (77). Intrafollicular injection of 1 ng of LH (NIH-LH-S16) into rabbit Graafian follicles induced luteinization (55). Binding studies with porcine granulosa cells (23) have led to the conclusion that granulosa cells of large preovulatory follicles have a much greater capacity to bind LH than granulosa cells from small follicles.

The requirements for the maintenance of luteal function differ among

species (83, 87). In general, the function of luteal cells under physiological conditions requires a gonadotropin complex which consists of various combinations of LH, FSH, and prolactin. The roles of FSH and prolactin are discussed later in this chapter (Sections III,C,1 and III,F,1). LH maintains the luteal cells and promotes progesterone secretion in several species (3). Continuous intravenous infusion of ovine LH into intact normal ewes starting the twelfth day after heat prolonged the life-span of the corpora lutea and increased progesterone secretion (58). Normal morphological appearance and progesterone secretion required LH when bovine luteal cells were incubated *in vitro* (40). Injection of ovine LH (NIH-LH-S16) into rabbit vesicular follicles increased progesterone secretion by 1000% within 5 minutes (84). Radioautographic studies (48) strongly suggest that the plasma membrane of luteal cells and thecal cells contain binding sites which are specific for LH.

## 2. Physiology of LH in the Male

The role of the gonadotropins in testicular function has recently been reviewed by Steinberger and Steinberger (118) and Nicoll (87). The prevalent view has long been that the testes secrete androgens in response to stimulation by LH. Although LH probably plays the dominant role, there is increasing evidence which indicates that FSH (Section III,C,2) and prolactin (Section III,F,2) may also stimulate testicular steroidogenesis.

LH stimulates androgen production by the testis both *in vivo* (33) and *in vitro* (22). Several recent studies support the long-held view that it is the interstitial cells (Leydig cells) of the testis which secrete androgens in response to LH. Aoki and Massa (2) showed that administration of LH induced changes in the ultrastructure of testicular Leydig cells which were seemingly related to secretory activity. Following LH administration, there was an expansion of the Golgi complex and rough endoplasmic reticulum and a marked depletion of lipid droplets. Radioautographic (30) and immunohistological (21) studies following *in vivo* hormone administration led to the conclusion that the interstitial cells are the major site of localization of LH within the rat testes. Radioiodinated human LH bound with high affinity to a rat interstitial cell preparation but not to a seminiferous tubule preparation following *in vitro* incubation (22). The specificity of the interstitial cell binding sites for LH is confined to LH and structurally similar HCG (22).

These high-affinity interstitial cell binding sites have been used as specific binding agents in radioligand-receptor assay systems which may be used for LH or HCG (22). These assays are somewhat more sensitive and precise than conventional bioassays. The receptor assays may also have

more biological relevance than the extremely sensitive radioimmunoassays since only that portion of the hormone molecule which normally interacts with the receptor is "recognized" by the interstitial cell binding sites. Dufau *et al.* (32) have employed testosterone production by collagenase-dispersed interstitial cells of the rat testis as the response in an *in vitro* bioassay for LH. This bioassay is sufficiently sensitive to permit measurement of the low levels of circulating gonadotropins in human plasma.

## C. FOLLICLE-STIMULATING HORMONE (FSH)

### 1. Physiology of FSH in the Female

The transformation of growing ovarian follicles into Graafian follicles appears to require gonadotropin stimulation considerably before an antrum is formed (44, 78). The prevalent view that *both* FSH and LH are *required* for the transformation of a growing follicle to a preovulatory follicle and subsequent estrogen secretion by the theca (44) now seems unlikely. Recent studies suggest that FSH alone is sufficient for complete development of the follicle. Nuti *et al.* (89) reported that the administration of low doses of highly purified ovine FSH brought about complete development of preovulatory vesicular follicles and increased serum estrogen levels in hypophysectomized female rats. Although FSH alone appears sufficient to stimulate the complete development of ovarian vesicular follicles, it is likely that under physiological conditions both FSH and LH play a role in follicular development (44). Kraiem and Samuels (59) found that FSH alone brought about a slight increase in several enzymes involved in steroid biosynthesis in the mouse ovary. However, FSH plus LH was more effective in increasing the levels of these enzymes.

Several recent studies suggest FSH effects upon the ovary are mediated primarily through the granulosa cells. Radioautographic studies of topically applied gonadotropins led Zeleznik *et al.* (129) to conclude that specific binding of radioiodinated human FSH was confined to the granulosa cells of rat ovarian follicles which were developed beyond the primary follicle stage. These workers have suggested that FSH may bring about the maturation of granulosa cells in order that follicles may respond to the endogenous LH which is secreted during the estrous cycle. Radioautographic studies of ovaries of immature 25-day-old rats showed that HCG binding (presumptive LH receptors) was confined to thecal and interstitial cells. Following two days of priming with highly purified rat FSH, HCG, binding was also observed in the granulosa cells. In addition,  $\beta$ -hydroxysteroid dehydrogenase activity required for conversion of pregnenolone to progesterone appeared in the ovarian granulosa cells following FSH stimula-

tion. Zeleznik *et al.* (129) suggested that the appearance of the presumptive LH receptors and  $3\beta$ -hydroxysteroid dehydrogenase activity in the stimulated granulosa cells may indicate the initiation of luteinization of these cells. The results of the studies of Zeleznik *et al.* (129) are consistent with the earlier observation of Channing and Kammerman (23) that granulosa cells obtained from large porcine follicles have a higher capacity for binding LH than granulosa cells harvested from small follicles (Section III,B,1).

Hardy *et al.* (50) examined the ultrastructural changes in mouse ovaries deprived of FSH and found that the most striking morphological feature was the diminished width and irregular appearance of the zona pellucida which separates the granulosa cells from the ovum. The physiological significance of this observation is uncertain.

The principal role of the preovulatory surge of LH (Fig. 5) is generally believed to be the induction of ovulation; however, the function of the concomitant surge of FSH remains unclear. This FSH surge may be an important part of an "ovulation-inducing hormone" complex (Section III,B,1), may stimulate the growth and maturation of future generations of follicles (44), or both.

Luteinization of the granulosa cells and subsequent secretion of progesterone is generally thought to be caused primarily by LH; however, it appears that FSH is also capable of initiating granulosa cell luteinization. Hypophysectomized immature rats, which were injected for four days with highly purified ovine FSH followed by an ovulatory dose of ovine FSH, had elevated plasma progesterone concentrations which did not differ from those of rats which had received an ovulatory dose of ovine LH (89). Following the intrafollicular injection of low doses of ovine FSH (NIH-FSH-S6), rabbit Graafian follicles luteinized (84). The ovarian output of estradiol increased approximately 300% within 20 minutes while the output of progesterone increased 1000% within 5 minutes of FSH administration.

Available evidence indicates that FSH is probably not required for the maintenance of luteal function in all mammalian species, but Greenwald has reported that FSH and prolactin are required to maintain the corpora lutea in the hamster (43). Channing (25) demonstrated that low doses of highly purified human FSH maintained monkey granulosa cells in the luteinized state for at least 16 days *in vitro*.

## 2. Physiology of FSH in the Male

Immunohistological and radioautographic studies showed that FSH localized primarily in the Sertoli cells within the seminiferous tubules of

the testes (118). Binding studies support these results since cell preparations from seminiferous tubules bound radiolabeled highly purified human FSH (76), whereas interstitial cells bound little of this hormone.

Available evidence indicates FSH is necessary for the final steps of spermatid maturation (118). Recent advances have provided some insight into the probable role of FSH in this maturation process. Hansson *et al.* (49) have postulated that FSH stimulates the Sertoli cells to produce and secrete an androgen-binding protein (ABP), which increases the binding and accumulation of androgens within the seminiferous tubules. They further suggested that ABP might serve to concentrate androgens for the androgen-dependent germinal epithelium and also increase the amount of androgen transported within testicular fluid to the androgen-dependent caput epididymis.

The long-held view that LH but not FSH stimulates the testes to produce androgens does not appear to be accurate. Sherwood *et al.* (111) demonstrated that low doses of highly purified ovine FSH stimulated the growth of the androgen-dependent ventral prostates and seminal vesicles of immature hypophysectomized male rats as effectively as the standard LH preparation NIH-LH-S1. This LH-like activity is not due to LH contamination. The enzymic removal of sialic acid, a procedure which inactivates FSH but not LH, destroys the ability of the ovine FSH preparation to stimulate both the ovaries and male accessory glands (46). This observation indicates that the ventral prostate bioassay used to measure LH is not specific for LH.

The sites of FSH-dependent androgen biosynthesis have not been clarified. There is some evidence that the Sertoli cells secrete androgens. Lacy and Pettitt (60) found that human seminiferous tubule preparations incubated *in vitro* produced testosterone and that the Sertoli cells within these preparations contained the morphological features required for steroid biosynthesis.

## D. HUMAN CHORIONIC GONADOTROPIN (HCG)

### 1. Physiological Effects of HCG in the Female

The structure of HCG is very similar to LH; therefore, as might be expected many of the physiological characteristics of HCG are similar to those of LH. Human chorionic gonadotropin stimulates the interstitial cells of the ovary, causes ovulation, brings about luteinization of the granulosa cells, maintains the functional life of the corpus luteum, and increases progesterin secretion from luteinized cells (24, 27, 85). Human chorionic gonadotropin, like LH, augments the action of FSH on ovarian growth;

this augmentation is used in the relatively sensitive and specific HCG-augmentation bioassay for FSH which is described in Chapter 5.

In addition, HCG apparently has slight FSH-like activity. Administration of relatively high doses of HCG to rats causes growth of ovarian follicles (27).

In the human, HCG prolongs the life-span of the functional corpus luteum during early pregnancy and stimulates the corpus luteum to secrete the progestins which are required to maintain the uterine endometrium. Hirono *et al.* (51) have shown that HCG acts directly on the median eminence to inhibit secretion of LH/FSH-releasing hormone and have suggested that HCG may inhibit ovulation during pregnancy in the human.

Radioiodinated  $^{125}\text{I}$ -HCG is widely used for radioautographic studies of the cellular localization of HCG or LH receptors (96) and also for the study of HCG and LH interactions with both ovarian (63) and testicular (22) receptors. Radioiodinated HCG has been reported to have a greater binding affinity for HCG (LH) receptors and to be more stable than radiolabeled human LH preparations (22). In addition, radiolabeled HCG apparently retains its biological activity (22).

The sites of action of HCG (LH) appear to vary with the stage of development of the ovary. Radioautographic studies of the ovaries of immature rats showed HCG bound predominantly to interstitial cells with more limited binding to thecal cells (129). Following stimulation of follicle growth with FSH, HCG binding was observed in the granulosa cells (129). In pseudopregnant rats HCG is predominantly localized in the luteal cells (96).

## 2. Physiological Effects of PMSG in the Male

Human chorionic gonadotropin stimulates the interstitial cells of the testes to secrete androgens. As previously mentioned (Section III,B,2), the interstitial cell binding sites have been used as specific binding agents in radioligand-receptor assay systems for HCG (22). Testosterone production of dispersed interstitial cells from rat testes is employed as the response in an *in vitro* bioassay for HCG and LH. This assay is sufficiently sensitive to detect 2 pg of HCG (32).

## E. PREGNANT MARE SERUM GONADOTROPIN (PMSG)

### 1. Physiological Effects of PMSG in the Female

PMSG has physiological characteristics similar to both LH and FSH. LH-like characteristics include stimulation of ovarian interstitial cells, in-

duction of ovulation, and luteinization of granulosa cells (19, 27). Like FSH, PMSG is a potent stimulator of ovarian growth and increases the levels of estradiol in the blood. The administration of a single high dose of PMSG (50 IU) to immature female rats caused a greater than tenfold increase in ovarian weight within 72 hours (112). The administration of a relatively low dose of PMSG (2.5–20 IU) caused ovulation and mating behavior in immature rats (27, 125). Wilson *et al.* (125) have recently found that the timing and pattern of changes of peripheral plasma concentrations of estradiol, progesterone, and LH in PMSG-primed immature female rats are similar to those seen in cycling adult rats before ovulation. The effectiveness of a single injection of PMSG appears attributable to its long plasma half-life which has been reported to be 26 hours in the rat (91).

A single injection of PMSG followed approximately 65 hours later by a single injection of HCG induces a state of pseudopregnancy in immature female rats. The large heavily luteinized ovaries obtained with this gonadotropin "priming" are employed for the ovarian ascorbic acid depletion bioassay for LH and the luteal cell homogenate protein binding assay for LH described in Chapter 5.

## 2. Physiological Effects of PMSG in the Male

Daily treatment of intact immature rats with large doses of PMSG (32 IU) stimulates the development of interstitial tissue and accessory glands (27).

## F. PROLACTIN

### 1. Physiology of Prolactin in the Female

For a detailed recent review of the gonadotropic and antigonadotropic effects of prolactin, the reader is referred to Nicoll (87). The influence of prolactin on the gonads is complex and remains poorly understood.

Prolactin has been reported to constitute part of the luteotropic complex required for the maintenance of the corpus luteum of several species including rats (35), rabbits (116), hamsters (42), cattle (10), and sheep (31). In most species the complex consists of prolactin and LH; however, in the hamster, prolactin and FSH are apparently required (43). In some species, including primates, the role of prolactin in luteal function seems doubtful.

Prolactin also has antigonadotropic effects in the rat. Surprisingly, prolactin appears to be luteolytic as well as luteotrophic in the rat (126). It

has been suggested that the relatively high levels of prolactin during proestrus, shown in Fig 5, may serve to destroy the old set of corpora lutea (87) In addition, prolactin inhibits LH induced follicular luteinization and PMSG-induced ovulation in the rat (52) Injection of prolactin into mature domestic chickens causes regression in gonad size and function (87)

## 2 Physiological Effects of PMSG in the Male

Until recently a function of prolactin in the male was not recognized Bartke (8) found that injection of prolactin into infertile male dwarf mice which had a genetic deficiency in growth hormone and prolactin rendered them fertile Bartke and Lloyd (9) found that prolactin administration caused a significant increase in the yield of spermatozoa in the testes of these dwarf mice The spermatogenic effect in mice appears to be mediated through the Leydig cells Hafiez *et al* (47) have reported that prolactin acts synergistically with LH to stimulate testosterone secretion by the testes in rats and mice Prolactin also has antigonadal effects in the male of several nonmammalian species (87)

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# 3 Role of the Nervous System in Reproductive Processes\*

William F. Ganong

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## I. Introduction

The nervous system is involved to varying degrees in almost every aspect of the physiology of reproduction. Reflexes integrated at various levels of the nervous system are involved in sperm transport, parturition, and lactation. Copulation itself is made up of a series of reflexes and reaction patterns integrated in a coordinated whole, and sexual behavior is manifestly a subject for psychological and neurophysiological investigation. Another major aspect of neural involvement is the regulation by the brain of gonadal function through hypothalamic regulation of anterior pituitary gonadotropin secretion. The brain exercises a controlling influence on the amount and type of pituitary gonadotropic hormones liberated into the circulation. The hormones act on the gonads to bring about, in both sexes, the state of readiness in the reproductive organs and the maturation of the germ cells necessary for successful procreation.

\* This chapter contains previously unpublished data from research supported by USPHS Grant AM06704.

Such preparation would, of course, be in vain if it were not associated, in both sexes, with appropriate sexual behavior. This behavior is known to be dependent on an adequate level of circulating gonadal steroids. Thus, the gonads are involved in a kind of "feedback" mechanism. The brain controls the secretion of the gonadotropins; the gonadal hormones are secreted in response to stimulation by these tropic hormones; and the gonadal secretions act back on the brain to initiate the behavior necessary for successful reproductive performance.

The brain not only regulates gonadotropin secretion in adulthood but it also is responsible for the timing and coordination of the increase in the secretion of the gonadotropins that brings about sexual maturation. There is evidence that puberty occurs when the brain becomes less sensitive to the feedback effects of gonadal steroids.

The actions of gonadal hormones on the brain have also been shown to play a key role in the development and differentiation of hypothalamic function. In rats, both the adult pattern of gonadotropin secretion and sexual behavior depend on the pattern of sex steroid secretion during infancy, and in other species hormones exert similar inductive effects during fetal life. Thus, brain-endocrine interrelations determine the development and sexual differentiation of the individual as well as reproductive capacity once sexual maturity has been attained.

The role of the nervous system in several reproductive processes and various aspects of brain-endocrine interactions are discussed elsewhere in this treatise. Neural mechanisms involved in parturition are discussed in Chapter 13 and the neuroendocrine reflex responsible for oxytocin-induced milk letdown is described in Chapter 14. The pronounced effects of light and the ancillary effects of other environmental stimuli on gonadotropin secretion are discussed in Chapter 22. In the present chapter, the basic neural substrates of copulation are briefly considered, and attention is focused on the brain-gonad relationship in adulthood, the mechanisms regulating the onset of puberty, and the inductive effects of sex steroids on the brain early in life.

To keep this chapter as short as possible, emphasis has been placed on reviews rather than original papers as references. Additional references to original work published before 1969 can be found in the two previous editions of this chapter (36, 38).

## II. Neural Substrates of Mating Behavior

Mating behavior may legitimately be divided into two components. It includes, first, activity consequent to the urge to copulate—the interest in

or drive to sexual congress. Second, it includes the act of copulation itself. Sexual interest and the instinctual mating drive basic to the preservation of the species depend on neural circuits in the limbic lobe of the brain and hypothalamus (3, 44). Copulation itself is made up of a collection of reflexes and reaction patterns, including erection, the necessary postural adjustments, the pelvic thrusts in the male, the lordotic adjustment of the pelvis in the female, ejaculation, and orgasm.

The reflex arcs and centers in the nervous system controlling the motor patterns of the sexual act have been studied in considerable detail. It is known, for instance, that most of the postural adjustments for coitus in both the male and female are integrated at the spinal level (1). In dogs with spinal cord transections, stimulation of the genitalia leads to erection and pelvic thrusts in males, and perineal stimulation produces elevation of the pelvis in the females.

Erection may be initiated in humans by purely psychic stimuli, but the reaction is primarily a reflex one, initiated by genital stimulation and integrated in the sacral segments of the spinal cord. The efferent pathway is parasympathetic. The motor fibers pass to the genitalia in a relatively well-defined bundle, and since these fibers are also involved in ejaculation, the bundle has come to be called the "nervus erigens." The vascular engorgement responsible for erection is produced in part by closure of the so-called small sluice channels within the corpora cavernosa, but the main factor involved is arterial dilatation with consequent compression of the venous drainage (37).

Ejaculation in the male is initiated by stimulation of the glans, the adequate stimulus being gentle friction and the efferent pathway, the internal pudendal nerve. It is appropriately divided into two parts, emission and ejaculation proper (37). The first event, emission, is the delivery of semen into the urethra. It is primarily a sympathetic response integrated in the upper lumbar segments of the spinal cord and produced by the impulses that reach the smooth muscle of the vas deferens and associated organs via fibers in the hypogastric plexus. Ejaculation proper follows emission and is the expulsion of the seminal fluid from the urethra. This response is primarily parasympathetic, but it also involves a contraction of somatic musculature, particularly the bulbocavernosus muscles, which aids the expulsion. It is integrated in the upper sacral and lower lumbar portion of the spinal cord, and the motor fibers pass through the internal pudendal nerves and the nervus erigens. Ejaculation can still occur after sympathectomy, or treatment with drugs which block sympathetic discharge, but because there is no contraction of the musculature around the bladder neck, the ejaculate usually spills into the bladder.

Genital and other changes occurring during intercourse in human fe-

males have been studied in considerable detail (66). Orgasm regularly occurs in less than one-half of the female population. When it does, there are rhythmic contractions of the vaginal wall. Impulses also travel via the pudendal nerves and produce rhythmic contractions of the bulbocavernosus and other pelvic muscles. The contractions may aid sperm transport, but are clearly not essential for it.

Uterine contractions may occur in response to a spinal reflex during coitus in the female. A neuroendocrine reflex involving the posterior pituitary may also be involved. There is considerable evidence that genital stimulation during coitus initiates reflex release of oxytocin from the posterior pituitary (32). Some investigators have argued that the oxytocin acts on the uterus to initiate a series of contractions which facilitate the transport of sperm from the vagina to the fallopian tubes. However, there are great variations in the rate of sperm transport in different mammalian species, and Fitzpatrick (32) concludes, after a thorough review of this subject, that there is no proof that oxytocin secretion is an essential physiological component in mating.

### III. Regulation of the Secretion of Pituitary Gonadotropins by the Nervous System

#### A. THE MECHANISM BY WHICH THE NERVOUS SYSTEM REGULATES PITUITARY SECRETION

Many lines of evidence indicate that the brain regulates gonadotropin secretion. Sexual cycles in animals are correlated with changes in the seasons, an observation which is difficult to explain except in terms of the intermediation of the nervous system between the environment and the endocrine system. Temperature and rainfall changes may be responsible in part for seasonal variations, but in birds and mammals fluctuation in the incident light is the major environmental factor involved (see Chapter 22). In certain mammalian species (e.g., the cat, the rabbit, the ferret, and the mink) ovulation occurs only after copulation, and this reflex ovulation has been shown to occur in response to afferent stimuli that converge on the hypothalamus from the genitalia, the eyes, the nose, and other organs (19). Gonadal abnormalities are seen in humans with brain pathology. Several detailed studies of the endocrine findings in brain disease (2, 5, 76) make it clear that amenorrhea, hypogonadism and, alternatively, precocious puberty are relatively common complications of disease processes involving the hypothalamus.

The effects of hypothalamic disease in humans have been duplicated



by experimental lesions in laboratory animals. Gonadal atrophy and inhibition of gonadotropin secretion have been observed following hypothalamic lesions in rats, cats, dogs, monkeys, sheep, and a variety of other species (24). The other pituitary hormones are under neural control as well. Thus, at least as far as these hormones and the hormones of their target glands are concerned, the endocrine system can be regarded as one of the effector arms of the nervous system (37).

The possibility that the hypothalamic control of the anterior pituitary is exerted via nerve fibers to the gland deserves mention. There are sympathetic nerve fibers from the superior cervical ganglia which reach the anterior lobe along blood vessels. Parasympathetic fibers also reach the gland by way of the greater superficial petrosal nerve. However, complete sympathectomy does not prevent ovulation in the rabbit or pregnancy in other species (48). Similarly, there is little evidence for and considerable evidence against the parasympathetic supply to the anterior lobe being of significance in the control of hormone secretion. Simple section of the pituitary stalk in female laboratory animals, provided it does not infarct the pituitary or interfere with its revascularization by the portal vessels, permits a return of normal estrous cycles in a relatively short period of time. This interval is too short a period for regeneration of nerve fibers, if such regeneration can, indeed, be expected to occur from the hypothalamus. Thus, it is unlikely that the nerve fibers to the adenohypophysis play any important role in the control of its secretory function.

There is, however, a unique vascular connection between the brain and the anterior pituitary. The blood supply to the hypothalamus and pituitary in mammals is derived from the carotid arteries and the circle of Willis, the anastomotic arterial ring at the base of the brain. Branches of these vessels form capillary loops which penetrate the median eminence (ventral portion of the hypothalamus overlying the pituitary) and the posterior pituitary. From these loops, the blood is channeled into sinusoidal portal vessels which pass down the pituitary stalk and end in capillaries in the anterior pituitary (Fig. 1). The portal vessels that originate from the median eminence are referred to as "long portal vessels" and those that originate from the neurohypophysis are referred to as "short portal vessels." The hypophyseal portal system is a true portal system that begins and ends in capillaries without going through the heart, and it provides a direct vascular pathway between the brain and the anterior pituitary gland.

The hypophyseal portal system is a constant anatomical feature in higher vertebrates (48). There was originally some confusion about the direction of blood flow in it, but direct observations in amphibians, rats, and ducks indicate that most if not all of the flow is from the brain to the pituitary (23, 97). In many species of mammals and in all birds studied,

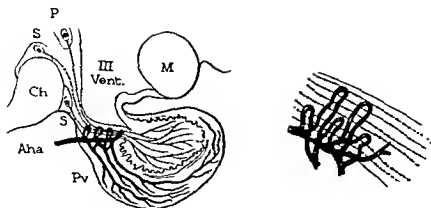


FIG. 1. Hypophyseal portal vessels. Left: Sagittal section of the hypothalamus showing a branch of the anterior hypophyseal artery (Aha) breaking up into capillary loops which penetrate the median eminence. The loops drain into the portal vessels (Pv) that end in capillaries in the anterior pituitary. Ch, optic chiasm; S, supraoptic nucleus; P, paraventricular nucleus; M, mammillary bodies; III VENT, third ventricle. Right: detail of capillary loops penetrating the median eminence. From Ganong (37).

the portal vessels provide essentially all the blood supply reaching the anterior lobe (23). The importance of vascular connections to the hypothalamus in the control of anterior pituitary secretion is demonstrated by the effects of cutting the normal connections between the pituitary and the hypothalamus. The portal vessels have a marked tendency to regrow (52), but if regrowth is prevented by the insertion of a plate of inert material between the hypothalamus and the pituitary, stalk section causes a marked decrease in FSH and LH secretion and an increase in prolactin secretion. Pituitary transplants "take" particularly well in the anterior chamber of the eye and under the capsule of the kidney. Such transplants are generally associated with marked gonadal atrophy. If the pituitary is retransplanted back to the median eminence region, the portal vessels regrow and there is a recrudescence of gonadal function. In animals with transplants remote from the hypothalamus, there is some return of gonadal function after relatively long periods of time. However, this does not mean that the anterior pituitary tissue is secreting autonomously, since the moderate rate of gonadotropin secretion in such animals is reduced by implants of testosterone in the ventral hypothalamus (94). Instead, the data indicate that anterior pituitary secretion is controlled by substances secreted by the hypothalamus into the blood stream.

Additional proof of the accuracy of the hypothesis that the anterior pituitary is controlled by blood-borne humoral agents is the extraction from hypothalamic and neurohypophyseal tissue of substances which modify anterior pituitary secretion. To date, eight of these hypothalamic

hormones have been identified (Fig. 2). Corticotropin-releasing hormone (CRH) regulates ACTH secretion; thyrotropin-releasing hormone (TRH) regulates TSH secretion; luteinizing hormone-releasing hormone (LRH) regulates LH secretion; and follicle-stimulating hormone-releasing hormone (FRH), together with LRH, regulates FSH secretion. The secretion of growth hormone and the secretion of prolactin are under dual control, the former being regulated by growth hormone-releasing hormone (GRH) and growth hormone-inhibiting hormone (GIH), whereas the latter is regulated by prolactin-releasing hormone (PRH) and prolactin-inhibiting hormone (PIH).

The structure of three of the hypothalamic hormones has been determined and they have been synthesized. TRH is a tripeptide, LRH is a decapeptide, and GIH, which is also called somatostatin, is a tetradecapeptide (37, 101). PIH may well be the catecholamine dopamine (69), but the others are probably polypeptides.

The availability of synthetic hypothalamic hormones has made it possible to produce antibodies to the hormones and use these antibodies to localize the hormones in brain tissue by immunocytochemical techniques. LRH and somatostatin are both localized in neurons that end in the external layer of the median eminence (105). Both are found in distinctive populations of intracellular secretory granules, the LRH granules (42, 80) being slightly larger than the somatostatin granules (79;

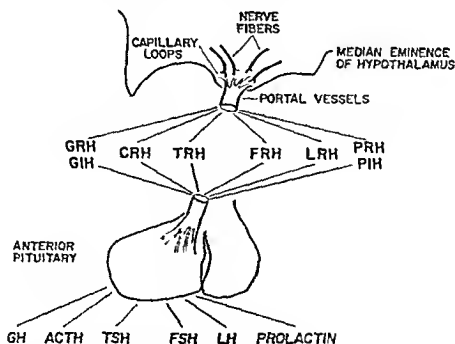


FIG. 2. Diagrammatic summary of the neurovascular control of the anterior pituitary. Control is exerted by the hypothalamic hormones secreted by nerve endings in the hypothalamus into the bloodstream and carried via the portal vessels to the anterior pituitary. For abbreviations, see text.

P. Goldsmith and W. Ganong, unpublished). It has also been claimed that there is some LHRH in ependymal cells (tanycytes), but the significance of this observation is uncertain (105). The cell bodies of the LHRH-secreting neurons are probably located in part in the arcuate nucleus and preoptic area. The cell bodies of the somatostatin-secreting cells are probably located in the ventromedial, arcuate, and medial preoptic areas, but their exact location is unknown. TRH-secreting neurons are probably located in the anterior and dorsal hypothalamus, and GRH-secreting neurons in the ventromedial nucleus, but the evidence for such localization is indirect (15). There is FRH activity in the arcuate nucleus, but LHRH stimulates the secretion of FSH as well as LH, and it is not possible at present to say if the activity is due to FRH or LHRH. Indeed, there may be only one gonadotropin-regulating hypothalamic hormone, LHRH. However, there is evidence for a separate FRH (67). PRH and PIH are discussed below.

What regulates the secretion of the hormone-secreting neurons? The hormones of the anterior pituitary, adrenal cortex, and gonads probably feed back to inhibit and, in some instances, to stimulate the secretion of the neurons. Impulses in the neural pathways that converge on the ventral hypothalamus also play an important role. The principal synaptic transmitters found in the median eminence include norepinephrine, dopamine, serotonin, acetylcholine,  $\gamma$ -aminobutyric acid (GABA), and various amino acids (15); it appears that most if not all of these substances affect anterior pituitary function (55). Epinephrine is also present in the hypothalamus (see below).

A major recent advance made possible by the discovery of a fluorescence technique for visualizing monoamines in tissue (73) has been the delineation of systems of neurons in the brain that secrete norepinephrine, dopamine, and epinephrine (51, 100). The noradrenergic neurons have their cell bodies in the locus ceruleus and other portions of the hindbrain, and their axons project to all parts of the brain and spinal cord. The neurons that innervate the ventral hypothalamus have many of their cell bodies in the medulla oblongata (75) and reach the hypothalamus via the ventral noradrenergic bundle. There are several systems of dopaminergic neurons in the brain, but the one that is the most directly concerned with endocrine function is the tuberoinfundibular system. Cell bodies of the tuberoinfundibular neurons are located in the arcuate nucleus and adjacent periventricular area, and their axons terminate in the external layer of the median eminence on or near the capillaries that coalesce to form the hypophyseal portal vessels. The cell bodies of the serotonin-containing neurons are located in the raphe nuclei of the hindbrain and their axons project to the limbic system and other parts of the forebrain.

When serotonin-containing pathways were mapped with the fluorescence technique, relatively few fibers were visualized entering the ventral hypothalamus. However, more recent experiments indicate the presence of a prominent serotonergic innervation of the suprachiasmatic nuclei, and there is a relatively large amount of serotonin in the median eminence (15). Epinephrine-containing neurons have been visualized by immunofluorescence techniques utilizing antibodies to phenylethanolamine-*N*-methyl transferase, the enzyme that converts norepinephrine to epinephrine. They have their cell bodies in the medulla oblongata and project to the hypothalamus (51). There are relatively few of these fibers, but the fact that they terminate in the hypothalamus suggests that they may play a role in the regulation of endocrine function.

Cholinergic pathways to the hypothalamus have not as yet been mapped in detail, and the morphology of the neurons that secrete GABA and the other amino acid neurotransmitters in the ventral hypothalamus is unknown. However, the availability of an immunocytochemical technique for localizing glutamic acid decarboxylase, the enzyme that catalyzes the formation of GABA (90), and work on the development of techniques for similar localization of the enzymes involved in the formation of acetylcholine make it likely that these pathways will be mapped in the near future.

## B. CONTROL OF GONADOTROPIN SECRETION IN THE MALE

When considering the regulation of gonadotropin secretion, the difference in the pattern of secretion between the two sexes makes it necessary to discuss the male and female separately. In adult males, the rates of FSH and LH secretion are more or less constant (24). In females, there is also steady secretion, but superimposed on this there is a cyclic increase, or burst of LH and FSH secretion that brings about ovulation.

Lesions in the posterior tuberal region that damage the arcuate nuclei (Fig. 3) have been shown to produce testicular atrophy in a number of different species. This atrophy is diffuse, and is associated with atrophy of the prostate, decreased sperm counts, and decreased secretion of LH and FSH (24).

Lesions which selectively inhibit FSH secretion might be expected to produce selective atrophy of the seminiferous tubules and inhibition of spermatogenesis without any change in the Leydig cells. Tubular atrophy with apparent maintenance of the Leydig cells has been reported in a few dogs and rats with lesions, but this combination has not been a consistent or reproducible finding. Androgen secretion in response to LH stimulation of the Leydig cells is capable of maintaining spermatogenesis

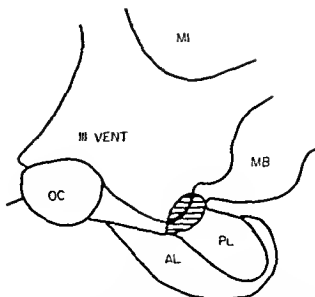


FIG. 3. Site of lesions which produce testicular atrophy and, presumably, inhibition of FSH and LH secretion in males. The striped area is the area common to lesions producing testicular atrophy in a series of male dogs. MI, massa intramedial; MB, mammillary bodies; PL, posterior lobe; AL, anterior lobe; OC, optic chiasm; III VENT, third ventricle. From Davidson and Ganong (26).

at or near normal levels in the absence of the pituitary (96), and this may explain the lack of consistent results. However, there is no clear-cut evidence for a center in the brain in males that affects FSH secretion without affecting LH secretion. There is also no good evidence for a discrete brain center concerned with the selective regulation of LH secretion in males.

In males, castration leads to an increase in circulating FSH and LH and to an increase in pituitary gonadotropin content in some species (24). In castrate animals and humans, doses of androgen which produce blood androgen levels in the physiological range decrease circulating LH (34). However, the nature of the testicular substance that inhibits FSH secretion is debated. Androgens do not decrease pituitary FSH content and large doses are needed to inhibit FSH secretion. In certain humans with marked atrophy of the seminiferous tubules but normal Leydig cells, FSH secretion is elevated (78). This suggests that the FSH-inhibiting substance is a product of the seminiferous tubules. It may be estrogen secreted by the Sertoli cells (24), but it may also be "inhibin," another putative hormonal product of the seminiferous tubules (34, 78).

The site at which androgens act to inhibit FSH and LH secretion appears to be the median eminence, although they may act at the pituitary level as well (24). Lesions in the posterior tuberal region of the median eminence in male dogs prevent the increase in pituitary FSH and LH con-

tent normally produced by castration and local implantation of androgen in the median eminence of male dogs produces testicular atrophy. Bogdanove (13) has argued that in this and other implantation experiments, one cannot rule out diffusion of the implanted steroid into the portal vessels and consequent action on the pituitary itself. However, Smith and Davidson (94) have presented evidence for a hypothalamic site of action which is difficult to refute. They studied hypophysectomized rats with pituitary transplants, in which testicular function had been maintained. When testosterone was implanted in the median eminence of these animals, testicular atrophy resulted. Control implants of cholesterol had no effect. The effect of implants could not have been due to a systemic absorption of the testosterone, since the accessory reproductive organs were not stimulated.

Support for the concept of a relatively autonomous gonadotropin-regulating center in the ventral hypothalamus comes from the studies of Halasz and his associates (47), who developed a technique for cutting all the nerve connections to the median eminence-arcuate region. This leaves an isolated island of ventral hypothalamic tissue in an otherwise intact animal. Following the operation in adult male rats, testicular weight and histology are maintained at normal levels.

The observations on the regulation of gonadotropin secretion in males are thus consistent with the view that there is a single hypothalamic regulatory area in or near the arcuate nucleus that is concerned with regulation of FSH and LH secretion. Androgens acting on this region inhibit LH secretion, and in adults, this negative feedback mechanism plus the mechanism regulating FSH secretion maintain the steady-state secretion of gonadotropins that is necessary for normal testicular function.

### C CONTROL OF GONADOTROPIN SECRETION IN THE FEMALE

As noted above, the female resembles the male in maintaining a tonic secretion of LH and FSH regulated largely by negative feedback, but differs in having superimposed on the tonic control cyclic bursts of LH secretion that trigger ovulation (Fig. 4). In spontaneously ovulating species, these bursts occur at regular intervals characteristic of the species. A surge of FSH secretion occurs at the same time, and in rats, but not in humans, there is also a midcycle increase in prolactin secretion. Estrogen secretion rises during the follicular phase of the menstrual or estrous cycle, with a peak at the time of the LH surge (Fig. 4). It then falls but rises again during the luteal phase of the cycle. Progesterone secretion is low throughout the follicular phase and rises during the luteal phase. The secretion of estrogen and progesterone both fall late in the luteal phase as

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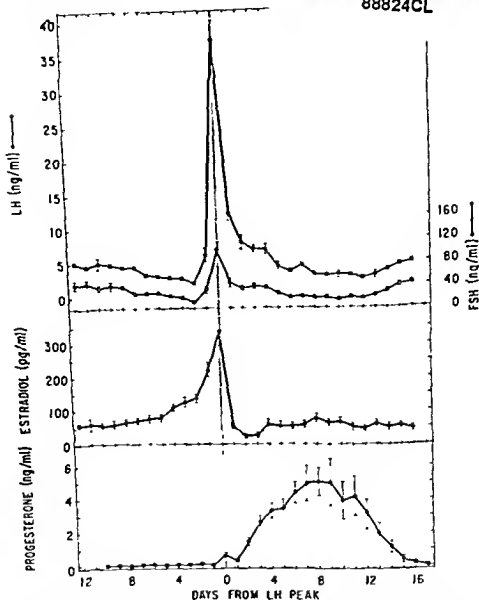


FIG. 4. Plasma concentrations of LH, FSH, estradiol, and progesterone throughout the normal menstrual cycle in Rhesus monkeys. Values are normalized to the day of the midcycle LH peak. From Knobil (59).

the corpus luteum regresses (luteolysis). Finally, a new follicle starts to grow and a new cycle commences.

Evidence that the mediobasal hypothalamus mediates the tonic secretion of FSH and LH comes from the observation that lesions of the arcuate nucleus produce diffuse ovarian atrophy. In addition, isolation of the mediobasal hypothalamus from all other neural connections to the rest of the brain does not alter the tonic secretion of FSH and LH (47). On the other hand, section of the pituitary stalk causes ovarian atrophy (48).

It is now clear that in spontaneously cycling mammals, the ovulatory



surge of LH secretion is triggered by the rise in circulating estrogen during the follicular phase of the cycle. Perhaps the most convincing evidence for this conclusion is the observation that prevention of the estrogen increase by administration of antibodies prevents the burst from occurring (31). No surges comparable to the ovulating bursts of LH secretion occur in ovariectomized animals without hormone treatment, and such increases can be produced in ovariectomized animals by injecting estrogen. Knobil and his associates have worked out in considerable detail the characteristics of estrogen stimulation in the monkey (59). The first effect of an injection of estrogen or the absorption of estrogen from a silastic implant is negative feedback with a decrease in circulating LH. However, if the plasma estrogen concentration exceeds 100 pg/ml and remains at this level for 36 hours, a burst of LH secretion is triggered. An estrogen rise of this magnitude occurs late in the follicular phase of the menstrual cycle in the monkey (Fig. 4). The increase in circulating LH then stimulates the secretion of estrogen, but estrogen secretion declines to low levels as soon as ovulation has occurred. The reason for the decline is unknown.

The negative feedback effect of estrogen on LH and FSH secretion appears to be exerted on the ventral hypothalamus, although it is difficult to rule out a direct action on the pituitary as well. Ovarian atrophy is produced by implants of estrogen in the median eminence, and estrogens and androgens produce changes in hypothalamic LRH and FRH activity (71, 82). Estrogen also acts on the brain more rostrally, in the supra-chiasmatic area, to trigger estrous behavior (49). The location of these two sites of estrogen action in the rat are shown in Fig. 5.

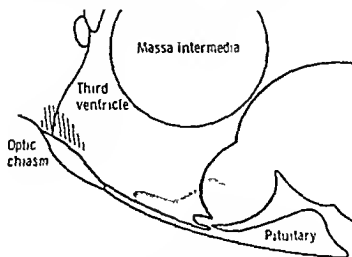


FIG. 5. Sites at which estrogens act on the hypothalamus. Estradiol implants in the infundibular region (shaded area) inhibit gonadotropin secretion, producing ovarian atrophy, whereas estradiol implants above the optic chiasm (striped area) induce estrous behavior. From Ganong (37).

In the rat, the part of the hypothalamus concerned with the cyclic burst of LH and FSH secretion can be separated from the area concerned with tonic secretion of the gonadotropins. This was clearly demonstrated by Halász and his associates, who showed that knife cuts separating the preoptic area from the mediobasal hypothalamus abolished ovarian cycles and produced a state of constant vaginal cornification (47). Lesions in the preoptic, suprachiasmatic, and related anterior hypothalamic areas produce a similar condition in rats and guinea pigs (33). The ovaries of these animals become full of follicles, but no ovulation occurs and no corpora lutea are seen. The vaginal epithelium becomes cornified and remains in that state. The condition produced by the lesions has been called the "constant estrus syndrome." The term is unfortunate since the "estrus" is confined to the vagina; the rats do not mate. The term "constant vaginal cornification syndrome" seems more appropriate. Enough estrogen is being secreted to maintain the uterus and cornify the vaginal mucosa. FSH and LH are secreted, since FSH is necessary for follicular development and LH is necessary for estrogen secretion from the follicles. The follicles are capable of ovulation since injections of LH promptly bring about luteinization. However, endogenous LH is not secreted in sufficient amounts to produce ovulation.

The lesions in the suprachiasmatic and preoptic area which produce the constant vaginal cornification syndrome appear to exert their effects through the basal arcuate area, since basal lesions block ovulation even before ovarian atrophy has a chance to develop (33). Thus, the arcuate nucleus, the nerve endings that secrete LRH, and the portal vessels form a final common pathway to the pituitary from the hypothalamus.

It is worth noting that a variety of other procedures produce the constant vaginal cornification syndrome, apparently by acting on the anterior hypothalamic LH regulating region. It can be produced, for example, by constant exposure to light (21). It also appears when female rats reach adulthood if they are injected with androgen in infancy (see Section V).

The situation in the monkey is different from the rat. In this species, knife cuts that create hypothalamic islands containing only the median eminence and the supraoptic, ventromedial, arcuate, and premammillary nuclei are associated with regular menstrual cycles (64). On the other hand, stalk section in the monkey produces gonadal atrophy and injection of antibodies to LRH causes gonadotropin secretion to decrease promptly to low levels (68), so the mediobasal hypothalamus and LRH secretion are necessary for both the tonic and the phasic secretion of gonadotropins by the pituitary.

Where does estrogen act to stimulate the midcycle burst of LH secretion? The simplest hypothesis would be that the LH burst is due to a

midcycle increase in LRH secretion, and that the site of the positive feedback of estrogen is the hypothalamus. Presumably, the feedback site in the rat would be the preoptic area or its environs, whereas the feedback site in monkeys would be located in the mediobasal hypothalamus. Evidence favoring this hypothesis is the recent observation that there is a distinct increase in the LRH content of hypophyseal portal blood in monkeys at the time of the LH surge (17). However, this does not prove that estrogen acts on the hypothalamus and there is a definite increase in pituitary sensitivity to LRH at the time of the LH surge. The literature on the direct effects of estrogen on pituitary responsiveness to LRH is confusing (53, 59), and additional research is needed to work out the details of the mechanism triggering the LH surge.

Impulses in afferent pathways converging on the hypothalamus trigger the ovulation-producing increases in LH secretion in animals that ovulate after copulation (14). In spontaneously ovulating species, the effects of light and other environmental stimuli on reproductive function (Chapter 22) indicate that in these animals impulses in afferents from sense organs also affect gonadotropin secretion. In rabbits, ovulation can be produced by stimulation of the uterine cervix with a glass rod. However, genital stimulation is not essential for ovulation since this occurs following coitus after local anesthesia of the vagina and neighboring regions. Ovulation can also occur after a great variety of sensory receptors have been destroyed. Neither removal of the olfactory bulbs nor destruction of the vestibular apparatus and cochlea by themselves block copulation-induced ovulation. Blinding is also ineffective. In cats, which also ovulate only after coitus, complete sympathectomy does not alter the response. It appears probable, therefore, that many stimuli converge on the hypothalamus and that no single afferent pathway is essential for LH release.

In rats, lesions just above the optic chiasm block the production of constant vaginal cornification by exposure to constant light (21). This raises the possibility that the inhibitory influence of light on the ovulation-producing release of LH might be mediated by pathways that leave the optic chiasm and enter the hypothalamus at this location. In ferrets, which can be brought into estrus in the winter by exposure to extra light, the effect of light is mediated via the optic nerves as far as the hypothalamus. However, interruption of the optic pathways beyond the hypothalamus has no effect on the response to light (18). This also suggests the existence of a direct retinohypothalamic pathway, although an effect via the accessory optic tracts is also a possibility. The existence of such a pathway has now been clearly demonstrated (50). In birds, light not only acts by way of the eye, but also penetrates the skull and appears to exert a direct effect on the diencephalon or the pituitary to increase gonado-

trophy secretion (4). The eyes are certainly the major receptors in mammals, but light does penetrate to the region of the diencephalon in rats, rabbits, dogs, and sheep (39).

The limbic system also sends afferents to the gonadotropin-regulating centers in the hypothalamus. Stimulation of the amygdala produces LH release and ovulation in two reflexly ovulating species, the rabbit and the cat (for references, see 38). It also produces ovulation in rats with the constant vaginal cornification syndrome produced by constant illumination, and stimulation of the septum has a similar effect. However, lesions of the septum and amygdala in rabbits do not affect reproductive behavior or copulation-induced ovulation, and the role of the amygdala in normal physiological control of gonadotropin secretion remains uncertain.

Progesterone has an inhibitory effect on LH secretion. This effect is shared by a variety of synthetic derivatives of progesterone, and is the basis of the worldwide use of orally active progestational agents as oral contraceptives. Basal LH secretion is relatively well-maintained during oral contraceptive therapy, but there is no LH surge at midcycle. The inhibitory effect appears to be exerted on the hypothalamus but an additional action directly on the pituitary is also a possibility (25). In monkeys progesterone also prevents estrogen from inducing an LH surge (59).

The impulses that trigger the midcycle surge of LH secretion in rats appear to be adrenergic. It was originally thought that dopamine was the mediator (58) but the results on which this conclusion were based have not been confirmed and considerable evidence has accumulated indicating that norepinephrine is the mediator at the endings converging on the final common path in the ventral hypothalamus (92). For example, stimulation of the preoptic area in rats produces an increase in LH secretion that is prevented by drugs that disrupt noradrenergic transmission, but is unaffected by drugs that disrupt dopaminergic transmission (56). The receptors on which the norepinephrine acts appear to be  $\alpha$ -adrenergic receptors. It has been known for a long time that drugs which deplete catecholamines and block  $\alpha$ -adrenergic receptors in the central nervous system prevent ovulation (91). In addition, ovulation can be initiated with norepinephrine in animals with anterior hypothalamic lesions (99) and there is evidence that the increase in circulating LH following ovariectomy (74) and the LH surge that triggers ovulation (57) are dependent on intact noradrenergic transmission in the central nervous system.

The situation in the monkey is quite different in that drugs which modify noradrenergic transmission regularly fail to block ovulation or the positive feedback response to injected estrogen (59). However, the catecholamines are not without effect because they are necessary for circadian oscillations in LH secretion to occur. Circadian oscillations are the regular

fluctuations in the high plasma LH level observed 2 or more weeks after ovariectomy. They occur at intervals of approximately 1 hour, and are promptly abolished by injections of estrogen. They also occur in rats, and in both species they are unaffected by section of the afferent connections to the mediobasal hypothalamus (9, 59). However, it appears that they are generated in nervous tissue because if the isolated pituitary is superfused with constant amounts of LRH the output of LH is steady rather than rhythmic (77). It has also been demonstrated that the circrchoral oscillations are apparently due to pulsatile secretion of LRH (17) and cannot be explained by a "short-loop" feedback of LH on its own secretion (59, 92).

The circrchoral oscillations are depressed and the mean LH level is reduced by injection of  $\alpha$ -adrenergic-blocking drugs in monkeys (7). In rats, they are also sensitive to drugs that prevent noradrenergic transmission. On the other hand, stimulation of dopaminergic receptors seems to inhibit the oscillations (28).

#### D. CONTROL OF PROLACTIN SECRETION

Prolactin has received considerable attention in recent years. Although bovine and other nonprimate prolactins have been isolated and their structure determined (65), it has been difficult to separate prolactin from growth hormone in primates. Furthermore, synthetic human growth hormone has innate lactogenic activity. At one point, it was even suggested that there was no separate prolactin in primates. However, primate prolactin has now been isolated and partially characterized, and immunoassays have been developed for human and monkey prolactin (35). These and other immunoassays have made it possible to investigate the details of the regulation of prolactin secretion.

In all mammals, prolactin secretion is tonically inhibited by the nervous system. Lesions of the median eminence, separation of the hypothalamus from the pituitary by section of the pituitary stalk, and transplantation of the pituitary to a site distant from the hypothalamus are all associated with an increase in prolactin secretion (30). Hypothalamic extracts contain a substance that inhibits prolactin secretion (PIH) but they also appear to contain a PRH (69).

Stressful stimuli and exercise increase prolactin secretion. There is a diurnal rhythm in prolactin secretion in humans, with an increase during sleep. The sleep increase is prolonged, reaching a peak 5-7 hours after sleep onset. Prolactin secretion increases during pregnancy and falls during the postpartum period if the young do not nurse. Estrogens increase prolactin secretion. Suckling or artificial stimulation of the nipple pro-

duces a prompt, marked increase in prolactin secretion (for references, see 35, 38). Nonlactating women when suckled by infants, exhibit mammary gland development and lactation. Suckling of virgin female rats by foster litters results in the induction of lactation and pseudopregnancy. The relation of prolactin to lactation is discussed in more detail in Chapter 14.

In some species, increased prolactin secretion and pseudopregnancy can be induced by copulation with a sterile male and by mechanical or electrical stimulation of the cervix (30, 38). Prolactin plays an important role in the development of pseudopregnancy.

Visual and tactile stimulation such as seeing or touching eggs or young in the nest may lead to prolactin release in birds. The prolactin facilitates subsequent incubation, crop sac development, and broody behavior in ring doves and domestic pigeons. Prolactin release in response to seasonal and environmental changes has also been suggested to be responsible for premigratory deposition of fat in passerine species of birds.

Prolactin secretion is decreased by drugs which increase the release of dopamine in the central nervous system, and its secretion is markedly stimulated by drugs which block dopaminergic receptors. Such blocking agents include pimozide and the phenothiazine tranquilizers, such as chlorpromazine and perphenazine. The fact that the tuberoinfundibular dopaminergic neurons end on the portal vessels in the median eminence raises the possibility that dopamine acts directly on the pituitary. McLeod and his associates (69) have found that dopamine can inhibit the secretion of prolactin from the pituitary gland *in vitro* in amounts that are probably in the physiological range. Furthermore, infusion of dopamine directly in the portal vessels inhibits prolactin secretion (98).

Can the prolactin-inhibiting activity of hypothalamic extracts be explained by their dopamine content? Shaar and Clemens (93) have presented evidence that they can. They found that treatment of hypothalamic extracts with monoamine oxidase abolishes their PIH activity, that removal of catecholamines from the extracts by absorption on alumina also abolishes their PIH activity, and that inhibitors of monoamine oxidase potentiate the PIH activity of the extracts. On the other hand, there is some evidence for a noncatecholaminergic PIH (29), and dopamine has not yet been detected with certainty in hypophyseal portal blood. Consequently, although there is considerable evidence that dopamine secreted into the portal vessels by the tuberoinfundibular dopaminergic neurons could be a physiological PIH, and possibly the only PIH, definitive proof of this hypothesis is not yet available.

It has also been established that procedures and drugs that increase the discharge of serotonergic neurons in the brain increase prolactin secre-

tion (60). There is a relatively large amount of serotonin in the median eminence (15), but it does not appear that serotonin acts directly on the anterior pituitary to increase prolactin secretion (8). In addition, it does not appear that the effect of serotonin on prolactin secretion is mediated by way of dopamine. For example, the increase in prolactin secretion produced by suckling is mediated via serotoninergic neurons, and suckling does not produce a rapid, primary increase in dopamine metabolism in the hypothalamus (102). Thus, it appears that serotonin stimulates the secretion of PRH.

The chemical nature of PRH is presently unknown. TRH stimulates prolactin secretion (101) and this stimulation is very marked in some species. However, suckling and other stimuli that increase prolactin secretion do not simultaneously increase TRH secretion, so it appears there is a PRH in addition to TRH.

The mechanism regulating prolactin secretion in birds is different from that in mammals (63). It is interesting that hypothalamic extracts from birds stimulate rather than inhibit the release of prolactin *in vitro*. Hypothalamic extracts from parent pigeons actively secreting crop milk have been shown to be more effective in stimulating prolactin release by pigeon pituitaries than extracts from young pigeons having no crop gland stimulation.

There is some information about the afferent paths to the hypothalamus that are involved in the regulation of prolactin secretion. Lesions of the reticular formation in the brain stem inhibit lactation (6). This effect may be due to interruption of the milk ejection reflex (see Chapter 14), but the possibility that such lesions also reduce the prolactin response to suckling should be considered. There is, in addition, evidence that olfactory nerve stimulation can inhibit prolactin secretion. This may be the basis of the "Bruce effect," the interruption of pregnancy that occurs in mice exposed to a cage recently occupied by a male mouse of another strain (16).

#### IV. Regulation of the Onset of Puberty by the Nervous System

In addition to the part it plays in the endocrine and behavioral aspects of reproduction, the brain is involved in the initiation of puberty. Precocious puberty occurs in children with tumors or infections of the diencephalon (54). The sexual development in these children includes spermatogenesis in the male and ovulation in the female, and is normal in all respects except its timing. Thus, it is a "true" precocious puberty, and

differs from "pseudoprecocious puberty," the condition in which secondary sexual characteristics develop without maturation of germ cells due to excessive secretion of gonadal steroids in children with conditions such as ovarian and adrenal tumors. True sexual precocity may occur in very young children, and cases of regular menstruation in 2-year-old girls have been reported.

Pineal tumors have also been reported to cause precocious puberty in humans (54). For unknown reasons, precocity in association with such tumors is found almost exclusively in males. It is generally associated with lesions that destroy the pineal body, but pineal tumors, because of their anatomical position, also compress the hypothalamus early in their growth (104).

The most complete experimental studies of the mechanisms regulating the onset of puberty have been carried out in rats (27, 45). In this species, the vaginal canal does not become patent until the time of puberty. In some rats, the vaginal smear is estrous when opening occurs, whereas in others the first estrus occurs 1-2 days later. The average normal age of vaginal opening is about 35 days. In male rats, the testes become mature and sperm heads appear in the seminiferous tubules at about 40 days of age. Motile sperms appear later and puberty occurs about 50 days of age (12).

The failure of puberty to occur earlier in female rats is not due to unresponsiveness of the tissues to gonadal steroids, or to unresponsiveness of the gonads to gonadotropins. Ovaries of immature animals function in the adult manner when transplanted into adults and injections of gonadotropins after the age of 20 days can cause ovulation and corpus luteum formation. Precocious vaginal opening and cornification of the vaginal epithelium can also be produced by injections of gonadal steroids. The pituitaries of immature animals contain gonadotropins, and these can be released by appropriate hypothalamic-releasing factors, since precocious puberty can be produced by injection of hypothalamic extracts (40). In addition, the pituitaries of immature animals are capable of supporting normal estrous cycles when transplanted under the hypothalamus in hypophysectomized adult female rats (52).

Changes in plasma gonadotropins, prolactin, and estrogens during sexual maturation have now been analyzed in considerable detail, particularly in the female rat (Fig. 6). Plasma prolactin is low early in life and rises the day before vaginal opening (83, 103). FSH secretion starts to rise at approximately 10 days of age and plasma FSH concentration is very high at 15 days of age (61). It falls steadily thereafter, reaching a low level by the time of the onset of puberty. Plasma LH is relatively low throughout (Fig. 6), although some authors report irregular bursts of LH secretion from the tenth to the twentieth day of age (62).



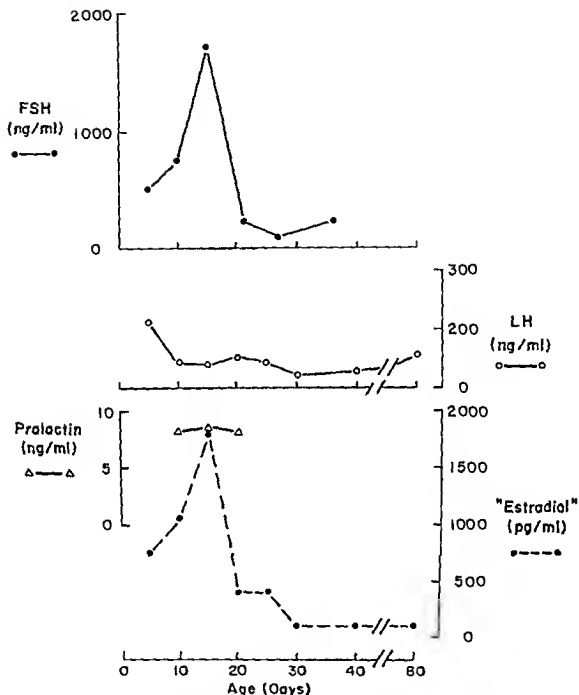


FIG. 6. Plasma concentrations of FSH, LH, prolactin, and "estradiol" in female rats of various ages. "Estradiol" is the material that reacts in a radioimmunoassay for estradiol, as reported by Rabii and Ganong (84). FSH values from Kragt and Dahlgren (61), LH values from Rabii and Ganong (84), and prolactin values from Rabii (83).

Twenty-four hours after ovariectomy, there is no increase in plasma gonadotropin concentration at the age of 5 days, but there is a brisk rise at 10 days, indicating the presence of negative feedback. There is a very large increase at 15 days of age. Thereafter, there is a steady decline in the increment produced by ovariectomy, and the increase produced in adulthood is only about one-half that produced at 15 days of age (84).

It has recently been shown that the plasma of immature rats contains quantities of a substance that cross-reacts with estradiol in the immuno-

assays usually used for this steroid (70, 84, 104). Ovariectomy causes a moderate reduction in the plasma concentration of this substance, but it is absent from the blood of ovariectomized animals that have also been adrenalectomized. It does not appear to be a biologically active estrogen because adrenalectomy plus ovariectomy does not produce any greater increase in plasma LH than ovariectomy alone. The physiological significance of the substance is unknown. It is also difficult to put changes in gonadotropin and "estradiol" secretion in rats in physiological perspective because estrogen levels are low in children, and LH and FSH secretion remain low until just before puberty (45). The data on other animals are too spotty to permit a detailed comparison to the rat and the human. However, it does seem clear that gonadotropins are capable of being secreted in sexually immature female rats, and that normally, they are not.

The failure of puberty to occur at a younger age also appears not to be due to a deficiency of hypothalamic-releasing hormones or the pathway by which they are transported to the pituitary. The LRH concentration in the hypothalamic tissue of 25-day-old rats is as great as that in adult female rats (86), and FRH is present in appreciable quantities as early as 10 days of age (61). Hypothalamic portal vessels appear in fetal rats at about 21 days of gestation, and the median eminence capillary network is nearly complete by the fifth day after birth (41).

Evidence that the brain is involved in the control of the onset of puberty in female rats is provided by the observation that hypothalamic lesions accelerate its onset by 4 to 5 days. Anterior hypothalamic lesions were first reported to have this effect (22), but some of these lesions may have produced the precocious onset of the constant vaginal cornification syndrome (see Section III, C) rather than true precocious puberty with regular cycles. Precocious puberty is also produced by lesions in the tuberal region that involve the arcuate nuclei.

Decreased illumination slows the onset of puberty, while increased light accelerates it (22). Light is known to affect the secretion of gonadotropins, presumably via the nerve fibers that leave the optic chiasm and enter the suprachiasmatic nuclei (50). The limbic system also may play some role in the regulation of puberty, since lesions in the amygdala have been reported to hasten puberty (22). However, Bloch and Ganong (11) and others (88) have been unable to confirm this observation. It has been claimed in the past that the effects of lesions are nonspecific and that any stress can produce acceleration of the onset of puberty. However, lesions in many portions of the nervous system other than the amygdala, the anterior hypothalamus and the tuberal region have been reported to be without effect on the onset of puberty (27).

The question of the role of the pineal body on the regulation of puberty (see 38 for references) is still debated because of reports that melatonin, the putative hormone of the pineal gland, decreases the frequency of estrous smears in rats exposed to constant light. However, melatonin has little if any effect on the onset of puberty when administered to immature rats. It has been claimed that serotonin, which is also present in large quantities in the pineal, antagonizes the action of exogenous gonadotropins on the ovaries of immature rats, but it has also been claimed that serotonin slows the onset of puberty when injected directly into the hypothalamus. In addition, no consistent, reproducible acceleration of the onset of puberty has been produced by pinealectomy, and in the absence of such an effect, it is difficult to argue that the pineal has any important role in the regulation of sexual maturation.

The accelerating effect of hypothalamic lesions on puberty in rats suggests the presence of some sort of hypothalamic mechanism that tonically inhibits gonadotropin secretion in immature animals. However, no inhibitory substances could be identified in hypothalamic extracts prepared from the brains of immature animals (40; R. Gellert and W. Ganong, unpublished). Lesions in brain tissue have zones of irritation around them (89), and it is possible that the lesions produce stimulation of the surrounding normal arcuate nucleus with the premature discharge of releasing hormones into the portal circulation.

Considerable attention has been focused on the role of feedback mechanisms in the control of the onset of puberty. The exact amount of circulating estrogen in immature female rats is uncertain because of the adrenal "estrogen" that is measured in the immunoassays (see above). However, the increase in plasma LH produced by ovariectomy and the decrease produced by a given blood level of estradiol are greater before than after puberty (22, 84). It has therefore been suggested that small amounts of gonadal steroids hold gonadotropic hormone secretion in check before puberty, and that puberty is due to a decline in hypothalamic sensitivity to the negative feedback action of the steroids. Bloch and Davidson (10) produced accelerated growth of the testes and accessory reproductive organs in immature male rats by implantation of an antiandrogen in the median eminence. Antiestrogens have been reported to produce accelerated vaginal opening as well as precocious ovulation in 30-day-old rats (20). The data on hypothalamic lesions causing precocious puberty could be explained by the feedback sensitivity hypothesis if the lesions destroyed part of the neural region responsible for negative feedback inhibition. However, the decline in the feedback sensitivity of the hypothalamus appears to start at about 15 days of age (see above), while precocious puberty does not occur until 30-35 days of age. In addi-

tion, the results of several experiments are difficult to reconcile with the hypothesis. For example, production of ovulation by injection of PMSG (pregnant mare serum gonadotropin) in immature rats is apparently due to a direct stimulatory effect of the gonadotropin on the brain. Estrogen has a similar effect; it not only acts directly to open the vagina, but it causes true precocious puberty (85). This latter effect is apparently due to an action on the brain, since it can be produced by implantation of estrogen in the hypothalamus (95).

In contrast to the female, it has not been possible to produce clear-cut precocious puberty in male rats with hypothalamic lesions. Since it takes 40 days for sperm to develop from spermatogonia in the rat, it would be difficult to produce any significant acceleration in the time of appearance of mature sperms even if spermatogenesis were stimulated early in life. A search for early activation of the Leydig cells might be more productive. Early attempts, using histological endpoints, were unsuccessful (M. Lima-Ostes and W. Ganong, unpublished), but a detailed study of the problem with measurement of circulating testosterone might be of value. In humans, the length of the spermatogenic cycle is 74 days, but sexual maturation does not normally occur for approximately 14 years. Consequently, there is plenty of time for precocious puberty to manifest itself, and precocious puberty in boys is a well-established syndrome (54).

## V. Effects of Hormones on the Development and Differentiation of the Brain

The brain resembles the reproductive organs in that its development, like theirs, is determined by the hormonal environment early in life. The female pattern of gonadotropin secretion and sex behavior is innate, but the male pattern develops at puberty if the brain is briefly exposed to androgen during fetal or neonatal life. Most of the experiments demonstrating these actions have been performed in the rat, a species in which the young are particularly immature at birth (43). If testes are transplanted to infant female rats, they do not ovulate when they mature. Instead, they develop constant vaginal cornification. Ovulation can be produced in such animals by injection of LH or, after progesterone priming, by hypothalamic stimulation. Thus the rats are able to secrete LH at a steady level like the male, but they are unable to produce the peaks of LH secretion necessary for ovulation. In males castrated at birth, transplanted ovaries show the female pattern of cyclic ovulation with luteinization of the ruptured follicles; but if the castrated rats with transplants are treated with androgens early in life, the male pattern of gonadotropin secretion

develops. A single dose of androgen as small as 10  $\mu\text{g}$  of testosterone on the fifth day of life in the female is capable of producing the male pattern of gonadotropin secretion in adulthood. Estrogens have no effect or they produce constant vaginal cornification. Consequently, it appears that the cyclic pattern of gonadotropin secretion seen in the female is the innate pattern, and that exposure to androgen early in life converts this pattern to the steady pattern of gonadotropin secretion seen in the male.

Pfeiffer, who did much of the early work in this field, believed that the androgen acted on the pituitary to make the pattern of pituitary secretion the male pattern (81). However, pituitaries transplanted from male fetal rats to hypophysectomized female adult rats maintain normal estrous cycles. Thus, the sex of the pituitary is not fixed, but depends on the sex of the brain under which it is located (49).

The early exposure of the brain to hormones also determines the pattern of sexual behavior that develops in adulthood. Female rats treated with testosterone when they are 5 days of age do not behave sexually as females when they reach adulthood; instead, they attempt to mount other females with greater than normal frequency and show increased male sexual behavior. Conversely, males castrated at birth show increased female sexual behavior although they continue to act as males as well (49).

The similarity of the action of androgen on brain development to its action on the development of the external genitalia is striking. In many species, androgen from the fetal testes causes the undifferentiated genital anlage to develop into male external genitalia. In the absence of androgen, female external genitalia develop regardless of genetic sex (46). It is worth noting, however, that the androgen effects on the genitalia occur earlier than the androgen effects on the brain. Therefore, it is possible to have normal genital development and abnormal brain development (49).

The neural effects of early exposure to steroids are most easily studied in the rat because in this species the changes can be produced by treatment after birth. In other species, treatment must be given *in utero*, usually by treating the mother. For example, female pseudohermaphrodite offspring of Rhesus monkeys treated during pregnancy with androgens have been shown to have abnormal sexual behavior in adulthood (44). There is also evidence for masculinization of behavior in girls exposed to high levels of androgens *in utero* (72). However, in primates, the pattern of gonadotropin secretion is not changed by early exposure to steroids, and androgenized monkeys and humans can menstruate regularly after sexual maturation.

It is pertinent to note that early treatment of female rats with large doses of steroids other than androgens produces the constant vaginal cornification syndrome in adulthood. Estrogens, corticoids, and even cho-

lesterol can all produce the syndrome (43). However, most steroids have the actions of other steroids if administered in sufficiently large doses. Consequently, it is unreasonable to expect complete specificity of the action of steroids on the brain. It has been postulated that estrogens act directly on the brain to masculinize it, and that androgens act by conversion to estrogens in the brain (87). However, this hypothesis has not been widely accepted.

It has also been reported that injections of reserpine, chlorpromazine or suspensions of thymus cells along with the androgen in early life prevent the constant vaginal cornification syndrome from developing in adulthood (43). The reasons for the blockade are unknown, but so far that matter is the mechanism by which the androgen exerts its masculinizing effect on the brain in the first place.

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# 4 Gonadal Hormones and Uterine Factors

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## I. Biochemistry of Androgens

### A. INTRODUCTION

#### 1. Basic Structure and Nomenclature\*

The androgens, estrogens, progesterone, and adrenal cortical hormones all are derived biosynthetically from cholesterol and possess a basic steroid

\* In the interest of brevity, symbols will be used where it is deemed appropriate. Androgens, estrogens, and progesterone will be referred to as A, E, and P, respectively. Symbols for other steroids are given in Table I.



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TABLE I

Nomenclature of Selected Androgens, Estrogens, and Progestins

Trivial name	Symbol	Systematic name
Androstenedione	A <sub>2</sub>	4-Androsten-3,17-dione
Testosterone	T	17 $\beta$ -Hydroxy-4-androsten-3-one
Dehydroepiandrosterone	DHA	3 $\beta$ -Hydroxy-5-androsten-17-one
Dihydrotestosterone	H <sub>2</sub> T	17 $\beta$ -Hydroxy-5 $\alpha$ -androstan-3-one
Estradiol-17 $\beta$	E <sub>2-<math>\beta</math></sub>	1,3,5(10)-Estratrien-3,17 $\beta$ -diol
Estradiol-17 $\alpha$	E <sub>2-<math>\alpha</math></sub>	1,3,5(10)-Estratrien-3,17 $\alpha$ -diol
Estrone	E <sub>1</sub>	1,3,5(10)-Estratrien-3-ol-17-one
Estriol	E <sub>3</sub>	1,3,5(10)-Estratrien-3,16 $\alpha$ ,17 $\beta$ -triol
Progesterone	P <sub>4</sub>	4-Pregnen-3,20-dione
Pregnenolone	P <sub>5</sub>	3 $\beta$ -Hydroxy-5-pregnen-20-one
20 $\alpha$ -Dihydroprogesterone	20 $\alpha$ -OHP	20 $\alpha$ -Hydroxy-4-pregnen-3-one
20 $\beta$ -Dihydroprogesterone	20 $\beta$ -OHP	20 $\beta$ -Hydroxy-4-pregnen-3-one
17 $\alpha$ -Hydroxyprogesterone	17 $\alpha$ -OHP	17 $\alpha$ -Hydroxy-4-pregnen-3-one
17 $\alpha$ -Hydroxypregnenolone	17 $\alpha$ -OHP <sub>5</sub>	5-Pregnen-3 $\beta$ ,17 $\alpha$ -diol

structure (Fig. 1a). Over 1800 biologically active steroids have been isolated from biological sources or are produced synthetically. Each of the above hormones is designated by a trivial name or by a systematic name which describes the chemical and stereoisomeric characteristics of the hormone. Lack of space does not permit a discussion of the chemical nomenclature and stereoisomerism of each of the gonadal hormones, but a brief treatment of this subject using androgen as a model is desirable. Androgens are named systematically (chemically) by reference to a parent hypothetical steroid structure, androstane (Fig. 1a and b), which is a completely reduced structure with 19 carbon atoms arranged in three six-membered rings (A, B, and C) and one five-membered ring (D). In the naturally occurring androgens substitutions in the ring system are an  $\text{=O}$  ( $\text{=O}$ ) or an  $\text{OH}$  ( $\text{OH}$ ) on carbon atoms 3 or 17 (Fig. 1c and d). Hence the androgens are named as substituted androstane, or if a double bond is present in one of the rings, as substituted androstene. The systematic termination is that recommended by the International Union of Pure and Applied Chemistry (IUPAC) (107, 32).

## 2. Stereoisomerism

The spacial orientations of the rings in the androstane molecule and of substituents on the carbon atoms determine the relative physiological activities of androgenic hormones on target organs and the union of these hormones with proteins and/or enzymes during their transport and cellular

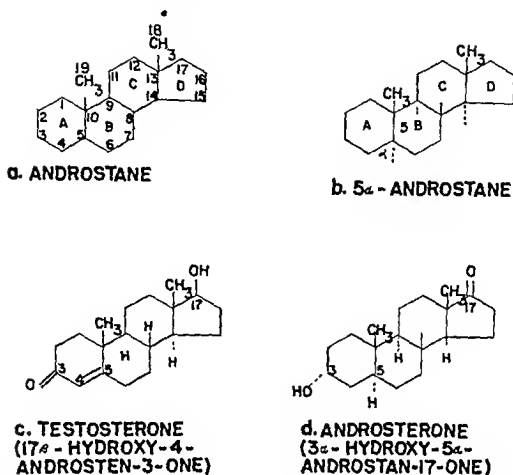


FIG. 1. Basic structure and nomenclature of androgens. Androstane (a) is the hypothetical parent steroid.

activities. The carbon atoms in the rings do not lie in a plane. They are oriented in space in the so-called chair form, and the substituents on the carbon atoms may be in the *cis* or  $\beta$  form (above the ring) or the *trans* or  $\alpha$  form (below the ring). Thus androstane exists in two forms: 5 $\alpha$ -androstane (Fig. 1) or 5 $\beta$ -androstane. In active androgens, rings A and B are in the *trans* relationship and are 5 $\alpha$ -androstane derivatives, or have a double bond in ring A between C-4 and C-5. Rings B/C and C/D are always in the *trans* spacial configuration in the biologically active androgens (Fig. 1c and d). The methyl groups ( $\text{CH}_3$ ) on carbon atoms 10 and 13 are fixed in the *cis* position in all steroid structures. The structures of two naturally occurring androgens are presented in Fig. 1 (c and d). Note that *cis* substituents are attached to the C atoms by solid lines and the *trans* substituents by broken lines.

## B. BIOSYNTHESIS OF THE ANDROGENS

The total biosynthesis of testosterone (T) from acetate by a variety of pathways in which cholesterol and the  $\text{C}_{21}$  intermediates  $\text{P}_3$  and  $\text{P}_4$  are implicated occurs in the testes and other steroid-producing tissues. The steps in the general biosynthetic pathway from cholesterol to  $\text{P}_4$  are shown

in Fig. 2. Pregnenolone is converted to  $P_4$  in the ribosomes of the testes by the enzyme isomerase (200).  $P_4$ , usually considered the female sex hormone because of its production by the ovary, and dehydroepiandrosterone (DHA), a product of the adrenal cortex, are precursors of testicular androgen. It is important to note that the ovary, adrenal cortex, and testis differ little in the type of steroid hormones produced; they differ in the quantities of the individual steroids synthesized and secreted.

In the rat, testicular steroid hormones are derived from cholesterol by a pathway leading to the production of  $P_4$  and isocaproaldehyde; the latter spontaneously forms isocaproic acid. Incubation of labeled cholesterol with a homogenate of rat testes yielded 5.2% as isocaproic acid and 3.7% as steroids; the latter fraction contained T,  $P_4$ ,  $P_5$ ,  $17\alpha$ -OHP, and  $20\alpha$ -OHP. The last three were present in quantities three times as great as T and  $P_4$  (58). The two-carbon ( $C_{17}$ ) side chain cleavage system is in the mitochondria and reduced nicotinamide dinucleotide phosphate is the most efficient cofactor. Abnormal human testes (interstitial cell tumor) converted  $P_4$  to T plus 4-androsten-3,17-dione ( $A_2$ ) while  $17\alpha$ -OHP was the major metabolite produced by normal testicular tissues (58).

One of the major pathways of T synthesis is as follows



Another pathway to T is

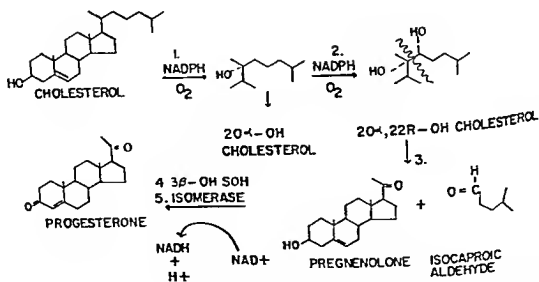


FIG. 2. Biosynthesis of progesterone from cholesterol. Steps 1-3 occur in mitochondria.

The synthetic pathways are shown in Fig. 3. Radioactive  $P_4$  was transformed to T by rat testicular microsomes in the presence of nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP). In the presence of testicular homogenates, T was formed from  $P_4$  with  $17\alpha$ -OHP and  $A_2$  as the intermediates. Only negligible amounts of  $17\alpha$ -OHP<sub>4</sub> and DHA were found (183).  $P_4$  is either converted to  $P_4$ , or may produce T by a pathway involving 5-androsten- $3\beta$ , $17\beta$ -diol-17-acetate and 5-androsten- $3\beta$ , $17\beta$ -diol.

A more detailed consideration of the enzymes and biochemistry related to biosynthetic pathways of androgens are presented in references (32) and (69).

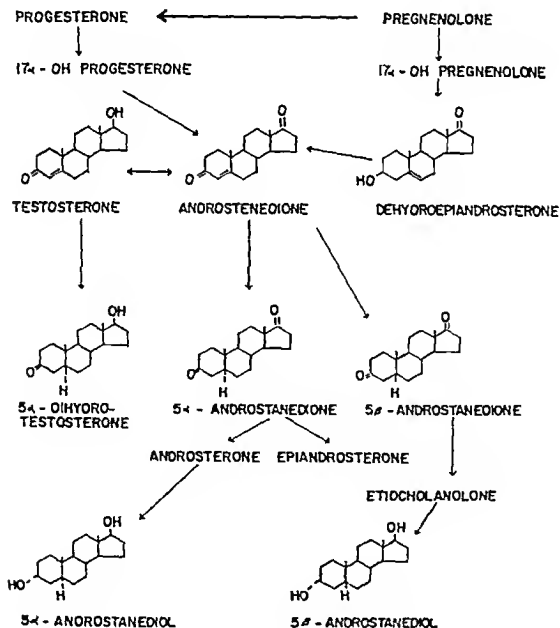


FIG. 3. Metabolism of androgens in domestic species.

Sulfoconjugated DHA, secreted in large quantities by the adrenal glands, undergoes direct metabolic changes in various endocrine and non-endocrine tissues. In the human ovary this sulfoconjugate contributes significantly to the biosynthesis of free androgens and estrogens (149). Skin tissue is also involved in sulfoconjugated  $C_{19}$ -steroid metabolism; the free compounds are found in human sweat extracts (150).

### C. CATABOLISM AND INTERACTIONS IN ANDROGEN-SENSITIVE TISSUES

In general we have pictured the steroid hormones as being produced by specific glands, then carried by the blood to target or metabolizing organs. In the metabolizing organs the steroids are catabolized to steroids having little or no biological activity, conjugated with sulfate or glucuronic acid to water-soluble forms, then excreted in the urine or feces. Recent evidence from studies of androgens indicates that target organs may require a transformation of the secreted form of the androgen. T, the principal androgen secreted by the testis, must be metabolized to several closely related steroids, principally dihydrotestosterone ( $H_2T$ ), prior to effecting the maximal biological stimulus (111). Owing to these recent findings, androgen metabolism can be discussed under two main categories: catabolism and metabolism in androgen-sensitive tissues.

#### 1. Catabolism

Testosterone, produced by the Leydig cells of the testis, is transported in the blood plasma attached to carrier proteins by a weak and reversible binding (61, 171, 176, 177). Serum albumin binds T in blood whereas corticosteroid-binding globulin binds both T-17 $\beta$ -sulfate and T-17 $\beta$ -glucuronide (191). Circulating  $A_2$  may be bound partially to plasma albumin and corticosteroid-binding globulin; some may be unbound.

The liver catabolizes the highly active testicular hormone T to  $A_2$  by the oxidation of the 17 $\beta$ -hydroxy group in the presence of 17 $\beta$ -hydroxysteroid dehydrogenase (17 $\beta$ -HSD).  $A_2$  is an intermediate which is further reduced to 5 $\alpha$ - and 5 $\beta$ -androstenedione. The two androstenediones are converted to androsterone (3 $\alpha$ -OH), epiandrosterone (3 $\beta$ -OH), and 5 $\beta$ -androsterone (inactive). These three are excreted in the urine or feces as soluble sulfates and glucuronosides. Testosterone may also be transformed to 5 $\alpha$ - and 5 $\beta$ -androstenediols by a reduction by specific  $\Delta^4$ -3 ketosteroid-5 $\alpha$ - and 5 $\beta$ -reductases and a specific 3 $\alpha$ HSD. The androstenediols are conjugated to glucuronosides prior to excretion. The relative

importance of the pathways depends upon the activity of the specific enzymes known to be present in hepatic tissue, prostate, skin, etc. (32). Figure 3 summarizes the catabolism of androgens.

Dehydroepiandrosterone is hydrolyzed to DHA sulfate by a steroid sulfatase (37) which may be converted to T in the testes both *in vivo* (37) and *in vitro* (52). Bailieu *et al.* (15) reported that DHA sulfate in testicular tissue of the boar occurs in quantities five times that of the free hormone. DHA is also a product of androgen catabolism and its sulfate is an important secretory product of the adrenals in both the male and female and may exert biological effects.

Research involving the qualitative and quantitative characterization of the excreted urinary metabolites of androgens has yielded knowledge of therapeutic and endocrinological importance, especially concerning the 17-ketosteroids (17-KS). The testes account for 30% while the adrenal cortex contributes the remaining 70% of the total urinary 17-KS (223). Androsterone and its less active  $3\beta$ -OH and  $5\beta$  isomers are the main urinary metabolites of T. DHA is the major 17-KS of adrenal origin. Methods are available for separating the testicular and adrenal 17-KS and for their quantitative determination, which are of value in discovering whether quantitative changes in the total 17-KS are due to testes or adrenal malfunction. Normal boars and castrates of both sexes produce 17-KS, whereas in normal sows no 17-KS is found in the urine (43).

Androgen metabolites are present in small quantities in male bovine urine (113, 222). Marker reported the presence of only two androgens in the bovine male: androsterone and DHA. Holtz, however, later found  $5\beta$ -androsterone and DHA but no  $5\alpha$ -androsterone after administration of T-propionate (113). Martin (126) injected 120 mg of radioactive [ $4\text{-}^{14}\text{C}$ ]T into a bovine male castrate and in the first 24 hours recovered radioactivity equivalent to 15 mg of the injected radioactive T. Two metabolites were isolated, namely, epitestosterone and  $17\alpha$ -hydroxy- $5\beta$ -androstan-3-one. Following the administration of P, to a pregnant cow, the following  $\text{C}_{19}$  steroids were identified in the feces:  $5\alpha$ -androstan-3,17-dione, 4-androsten-3,17-dione, and 1,4-androstadiene-3,17-dione (136). This was the first report of the isolation of the 1,4-androstadiene-3,17-dione from natural sources. The physiological importance of the conversions is uncertain. Owing to paucity of information, metabolites in the bile and enterohepatic cycling of steroids will not be discussed.

#### D. METABOLISM IN ANDROGEN-SENSITIVE TISSUES

Until recently, it has been generally assumed that steroid hormones were not metabolized in target organs (androgen-sensitive tissues) upon



which they acted biologically. Since the steroid hormones are protected from general catabolic processes by hormone-binding proteins in the blood and the liver, further metabolism in the androgen-sensitive tissues was unexpected (111). The use of  $^3\text{H-T}$  and other radioactive steroids has aided in studies of the disposition of androgens among various tissues. Williams-Ashman and Reddi (219) cite a number of reviews in which various aspects of this topic are considered in more detail. Injection of labeled T and  $\text{A}_2$  results in a selective uptake of these hormones by many androgen-sensitive organs including the prostate gland and seminal vesicles of mammals and the preen glands of ducks. In the female, certain male heterosexual remnant tissues also concentrate T *in vivo* (33, 221). Muscle tissues of the male do not selectively bind labeled androgens (137, 221). Within minutes after the injection of labeled T, the major metabolite produced in rat ventral prostate was  $\text{H}_2\text{T}$  (33). The cytoplasm of the prostate contained, in addition to  $\text{H}_2\text{T}$ , androsterone and some T. Only  $\text{H}_2\text{T}$  and smaller amounts of T were recoverable from prostatic cell nuclei for periods up to 2 hours. Only the prostate, seminal vesicle, preputial gland, kidney, and blood plasma (small quantities) contained free labeled  $\text{H}_2\text{T}$ . T is converted to  $\text{H}_2\text{T}$  in other androgen-sensitive tissues including the epididymis of rats (72), the comb, wattles, and coccyeal gland of chicks (72, 221), and the preputial glands of both male and female rodents (72). Gomez and Hsia (76) and Wilson and Walker (220) cited human skin as another androgen-sensitive tissue capable of converting T to  $\text{H}_2\text{T}$ . The rates of conversion were especially high with skin specimens from the scrotum, prepuce, labia major, and clitoris. Wilson and Walker (220) reported a progressive decline in the  $\text{H}_2\text{T}$  formation in the prepuce of adult men. Apparently, the reductases involved in the transformation of  $\text{H}_2\text{T}$  from T are stereospecific in their transfer of hydride ions from NADPH: the  $5\beta$ -reductase transfers this ion from the A position of NADPH to the  $5\beta$  position of T whereas the  $5\alpha$ -reductase transfers the ion from the  $\beta$  position of NADPH to the  $5\alpha$ -position of T (2).

The kidney appears to utilize T as the intracellular androgen whereas all other androgen-sensitive tissues utilize  $\text{H}_2\text{T}$ . Mowszowicz and Bardin (143) found an active 3-ketoreductase ( $3\alpha$ -HSD) in mouse kidney resulting in the formation of  $5\alpha$ -androstan- $3\alpha,17\beta$ -diol and little  $\text{H}_2\text{T}$  from T.

The discussion of the receptor hypothesis by which androgens are bound to proteins in the cytoplasm and nuclei of androgen-sensitive organs will be described briefly and in general terms here. In Section III differences present in various organs and tissues of importance to reproductive physiology will be discussed.

The receptor hypothesis, in general, postulates that the specificity of the nuclear binding of  $H_2T$  is controlled by the nature and presence of cytoplasmic receptors and by factors present in nuclear chromatin, perhaps in a DNA-associated nonhistone protein (65, 111, 122). The number of potential binding sites for  $H_2T$  in the nucleus has been reported to be ranging from 2000 (65) to 6000 (122). Saturation of these sites prevents further binding in the nucleus. Administration of T to castrate rats induces stimulation of protein synthesis in prostate nuclei. This suggests that androgens may influence the rate of synthesis of the nuclear proteins which regulate the binding of cytoplasmic receptors in chromatin (122).

In the rat prostate gland the nuclear binding of  $H_2T$  is similar to the mechanism of nuclear binding of other steroids, except for the fact that the naturally secreted hormone, T, must be converted to  $H_2T$  prior to binding. T is also converted to  $5\alpha$ -androstane- $3\beta$ , $17\beta$ -diol in the cytoplasm. This is bound to prostate microsomes but is not retained in the nucleus (111).

Two cytoplasmic receptors, complex I and complex II, exist, the former controlling retention of complex II in the nucleus, but the mechanism has not been elucidated.

A number of antiandrogenic substances have been described, but the mechanism whereby they lower or prevent androgenic activity is not understood. A  $6\alpha$ -bromo- $17\beta$ -hydroxy- $17\alpha$ -methyl-5-oxo- $5\alpha$ -androstane-3-one (BOMT) selectively suppresses the nuclear binding of  $H_2T$  in androgen-dependent tissue *in vitro* and competes effectively for the specific, high-affinity binding sites for  $H_2T$  in the cytoplasm of the rat prostate gland and its transfer into chromatin in a reconstituted cell-free system (123). BOMT also antagonizes the stimulation of RNA polymerase activity in the prostate gland after administration of T *in vivo*. It has no effect on the rate of formation of  $H_2T$ . Many aspects of the mechanism of action of androgens at the cellular level are unknown and require further investigation.

## E. SECRETION OF ANDROGENS

### 1. Sources

The Leydig cells in the testis produce the chief naturally occurring androgen in the male. That the ovaries produce androgenically active secretions has been demonstrated by a number of early investigators, but no pure androgen has been isolated from ovarian tissue (56).

Unlike the ovaries, androgens have been isolated from the adrenal cor-

tex by extraction. In the human female with adrenocortical hyperplasia, androgens are present in the urine in high concentration. Decreased amounts of urinary androgens are present in the urine of patients with adrenocortical hypofunction. Androgens are also present in the urine after gonadectomy.

## 2. Control Mechanisms

In animals lacking a definite breeding season, androgen production and secretion is continuous, with minor variations after puberty. In some domestic animals a breeding season occurs but is less obvious in the male than in the female. A number of stimuli in vertebrates cause gonadotropin release which, in the male, acts upon the testis and induces the production and release of androgens. Nothing is known about the capacity of different testicular cells for androgen storage. Eik-Nes (62) has reported an instantaneous increase in the concentration of T and precursors of T in spermatic venous blood of the dog. That gonadotropin secretion is controlled by pituitary active neurohumors of hypothalamic origin is now well established (225). Crude hypothalamic extracts and gonadotropin-releasing hormone (GnRH) of natural or synthetic origin increased serum LH in sheep (139). The effect of GnRH on release of LH in the bovine *in vivo* and *in vitro* has been investigated (140). Serum LH concentration in bulls prior to GnRH treatment averaged 1.1 ng/ml and increased to peaks of 9, 19, and 39 ng/ml after 10, 40 and 160  $\mu$ g GnRH, respectively. Serum LH in heifers behaved similarly in response to GnRH treatment. LH rose threefold in the effluent media from steer pituitaries continually superfused with GnRH after 1-minute exposure to 1 ng purified porcine GnRH/ml medium. GnRH did not release LH in quantities characteristic of the ovulatory surge, which suggested that a single surge of releasing hormone is not the exclusive event which induces the pre-ovulatory surge of LH.

In a more recent report (140), T increased about threefold in response to the increased LH release after GnRH in 6-month-old bulls but not in 2- or 4-month-old bulls. Unlike T, A<sub>4</sub> increased significantly at all ages, and the pattern of the A<sub>4</sub> response to GnRH generally was similar to that for LH. After further work (141), it was concluded that GnRH causes LH to increase after castration and the increase is not reversed by T. In addition, LH is released episodically in bulls, and peaks of LH normally are closely followed by increased T. Despite increased serum LH after GnRH at all ages and increased T after GnRH in 6-month-old bulls, testicular size, testicular sperm numbers, and epididymal sperm numbers were unaltered at 60 days after GnRH treatment.

## II. Biochemistry of Progesterone and Estrogen

### A. INTRODUCTION

In 1929, Corner and Allen (46) reported that an extract of the corpus luteum (CL) caused a proliferation of the uterus. the active principle was identified simultaneously by three groups of workers (5, 38, 192) and named progesterone ( $P_4$ ). It contains the perhydrocyclopentanophenanthrene ring with a two-carbon side chain at the C-17 position and oxygen functions at C-3 and C-20. By 1957, the hormone had been identified in ovarian tissue, adrenal tissue (18), placenta (170), and blood (185).

Besides being a hormone,  $P_4$  is an intermediate in the synthesis of androgens and estrogens. Four estrogens ( $E$ 's) have been found in the steroid-forming tissues of the cow, ewe, and sow: estrone ( $E_1$ ), estradiol- $17\beta$  ( $E_{2-\beta}$ ), estradiol- $17\alpha$  ( $E_{2-\alpha}$ ), and estriol ( $E_3$ ). (Equilin and equilenin occur also in the pregnant mare.) The  $E$ 's differ from other steroids; the A ring has three double bonds and no angular methyl group at C-10. There is a hydroxyl group at C-3 that is phenolic in character, thus  $E$ 's are soluble in alkaline solution.  $E$ 's are present in blood by a factor of 100- to 1000-fold less than  $P_4$ , yet this quantity of  $E$  exerts a biological effect due to its greater potency per unit weight.

Allen and Doisy, using the vaginal smear technique, showed that follicular fluid could produce symptoms of estrus in spayed rats and mice, thus giving evidence of the existence of a hormone (152). Small quantities of the hormone were available for study until the report of Aschheim and Zondek that the hormone was present in human pregnancy urine in much larger quantities (152). By 1931,  $E_1$  had been isolated in crystalline form from urine and its physical and chemical properties described (152). In 1936, MacCorquodale *et al.* extracted the follicular fluid contained in four tons of sow's ovaries, in the search for an ovarian  $E$  (152)  $E_{2-\beta}$  was identified, followed by  $E_1$ .  $E_1$  and  $E_{2-\beta}$  were identified in human follicles and CL in 1959 (224),  $E_{2-\beta}$  and  $E_{2-\alpha}$  in cow follicular fluid in 1958 (206) and  $E_1$  in 1962 (188), and  $E_{2-\beta}$  and  $E_1$  in follicular fluid of mares in 1962 (186). Reference (152) summarizes much of the early work.

### B. BIOSYNTHESIS OF PROGESTERONE

Elucidation of the synthetic pathway leading to  $P_4$  is based mainly on studies of the CL and adrenal cortex. The similarity of enzyme systems, their subcellular sites, and tropic hormone control makes one realize that one pathway is probably common to all steroid-producing tissues. This

pathway is shown in Fig. 2. The source of cholesterol is mainly the plasma (193) although the CL possesses all the enzymes required for synthesis of cholesterol from acetate (88) and does produce some cholesterol. Homogenization abolishes the incorporation of  $^{14}\text{C}$ -acetate into cholesterol (174). The reactions leading to  $\text{P}_s$  occur in the mitochondria (83, 105). In mitochondria, the side chain of cholesterol reacts with a multicomponent enzyme system that utilizes oxygen and NADPH to hydroxylate and cleave the chain between C-20 and C-22 of cholesterol. The rate-limiting step is hydroxylation of cholesterol at the C-20 position which can be stimulated by luteinizing hormone (LH), except in a cell-free system. Normally the side chain cleavage system is membrane bound. The end product of the mitochondrial system,  $\text{P}_s$ , is then free to traverse the membrane to be utilized for the synthesis of hormones by the various microsomal enzyme systems.

$\text{P}_s$  is the precursor for the steroid hormones produced by the adrenal gland (103), placenta (154), testicular tissue (200), and the various tissues of the ovary (175). The enzymes that convert  $\text{P}_s$  to active hormones are found in the endoplasmic reticulum.  $\text{P}_s$  is converted to  $\text{P}_4$  by means of a  $3\beta$ -hydroxysteroid dehydrogenase ( $3\beta$ -HSD) and isomerase in the testes (200) and the ovary (175). Once  $\text{P}_4$  is synthesized it may be either secreted as in the CL or retained as an intermediate for other hormones as in testes.

### C. CATABOLISM OF PROGESTERONE

The catabolic pathways are shown in Fig. 4. A number of tissues contain  $20\alpha$ - and  $20\beta$ -HSD. The ovary itself catabolizes  $\text{P}_4$  to either  $20\alpha$ - or  $20\beta$ -dihydroprogesterone. The ovary of the cow and sow produces  $20\beta$ -OHP, while the ewe's ovary produces  $20\alpha$ -OHP (75). The ovarian enzyme seems to be significant in the control of  $\text{P}_4$  synthesis in the rat ovary (215).  $\text{P}_4$  levels fall before parturition which is due to the redirected metabolism of  $\text{P}_s$  to  $20\beta$ -OHP.  $20\alpha$ -HSD is under hypophyseal control during early pregnancy and later shifts to the placenta (216).

In target tissues, the 4-ene reductases degrade  $\text{P}_4$  to  $5\alpha$ - and  $5\beta$ -pregnanediones which are then reduced by  $3\alpha$ - and  $3\beta$ -HSD to  $3\alpha$ - and  $3\beta$ -pregnanolones. Further reduction occurs at the C-20 ketone group to form the  $5\alpha$ - and  $5\beta$ -pregnanediols. Eight possible isomers of pregnanediol may result. A scheme for the catabolism of  $\text{P}_4$  is shown in Fig. 4.

#### 1. Metabolites in Urine

Normally,  $\text{P}_4$  is not in the urine. Its major urinary metabolite,  $5\beta$ -pregnane- $3\alpha,20\alpha$ -diol, has been found in many species, among them being

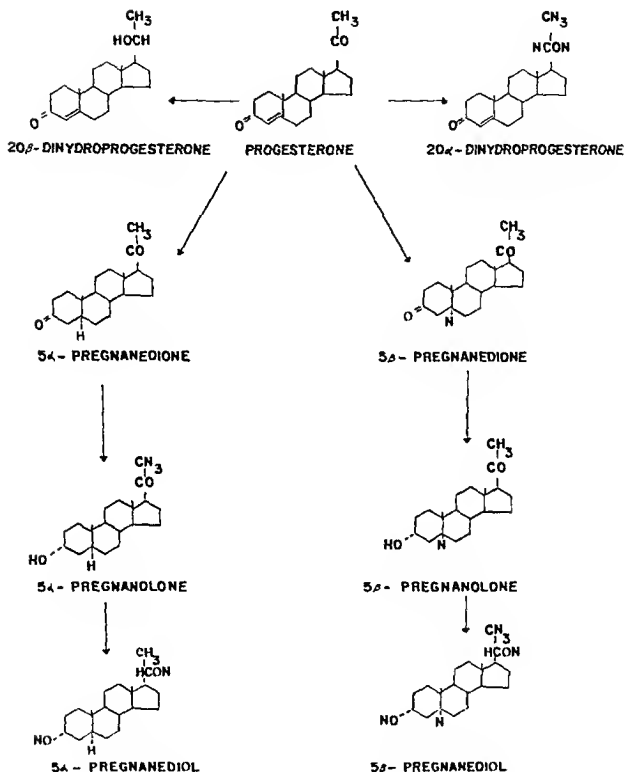


FIG. 4. Catabolism of progesterone in domestic species.

the sow (129), mare, and goat (112). Other compounds isolated from the urine have been: 5 $\alpha$ -pregnanediol and 5 $\alpha$ - and 5 $\beta$ -pregnanolone (sow; 129, 180), and the 3 $\alpha$  and 3 $\beta$  epimers of 5 $\alpha$ -pregnane-20 $\beta$ -diol (mare; 124, 125). When  $^{14}\text{C}$ -P<sub>4</sub> was administered to dairy cows, 3% of the radioactivity was recovered in the urine, and 50% in the feces (218). It ap-

pears that  $P_4$  is converted to androgens in the liver and excreted via the feces in ruminants (138).

## 2. Metabolites in Target Tissues

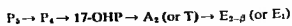
Most of the metabolic studies of target tissues concern the human, rat, and pig. Wickmann (214) reported the distribution of metabolites in rat myometrium and found  $\Delta^4$ -5 $\alpha$ -reductase and 3 $\alpha$ -HSD concentrated mainly in the nuclear-myofibrillar and mitochondrial fractions. In the supernatant, 20 $\alpha$ -HSD activity was detected. In an *in vivo* time-lapse study,  $P_4$  was first converted to 5 $\alpha$ -pregnane-3,20-dione. Within 1 minute, 3 $\beta$ -hydroxy-5 $\alpha$ -pregnane-20-one appeared, and to a lesser extent, 20 $\alpha$ -OHP. Finally 5 $\alpha$ -pregnane-3 $\alpha$ ,20 $\alpha$ -diol appeared. No 5 $\beta$ -pregnanes were observed in the rat uterus.

In human endometrium, 5 $\alpha$ - and 5 $\beta$ -pregnanes and 20 $\alpha$ -OHP have been readily detected along with small amounts of the pregnanediols (45). There is no evidence for 17-hydroxylation, cleavage of the side chain, or aromatization to estrogenic compounds. A total of fourteen catabolites of  $P_4$  have been identified. The rate of catabolism in the myometrium is much less than in the endometrium (36) with less than one-half as many catabolites being produced.

In an *in vitro* study of porcine endometrium collected on day 9 or 10 of the estrous cycle, and incubated with  $^{14}\text{C}$ - $P_4$ , Henricks and Tindall (90) reported eleven reduction products. Two of these were 5 $\alpha$ -pregnane-3,20-dione and 3 $\beta$ -hydroxy-5 $\alpha$ -pregnane-20-one. On the basis of chromatographic mobilities in three thin-layer systems, 20 $\beta$ -hydroxy-5 $\beta$ -pregnane-3-one and 20 $\alpha$ -OHP were reported. In further work in this laboratory using endometrium from day 16 or 17 of the cycle (unpublished data), catabolites from the previous study were identified as well as 3 $\alpha$ -hydroxy-5 $\alpha$ -pregnane-20-one and 5 $\alpha$ -pregnane-3 $\alpha$ ,20 $\alpha$ -diol. Thus it appears that in all three species, human, rat, and pig, the 5 $\alpha$  reduction series is the preferred route of catabolism in the uterus.

## D. BIOSYNTHESIS OF ESTROGENS

Androgens are the immediate precursors of the estrogens as shown in Figs. 5 and 6. The principal precursors are T,  $A_2$ , and DHA. This completes the biosynthetic pathway



From a variety of data based on the use of organ perfusions, *in vitro* incubations, direct extraction of isolated tissues, and *in vivo* studies,  $E_2$ 's

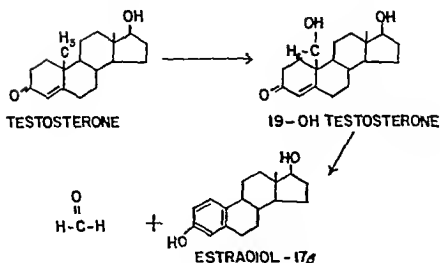


FIG. 5. Oxidation of C-19 methyl group of testosterone to form estradiol-17β and formaldehyde.

can be formed by the ovary placenta, adrenal, and testes (57). In most species, significant quantities are not produced outside the ovary and placenta, except in special cases such as adrenal or testicular tumors.

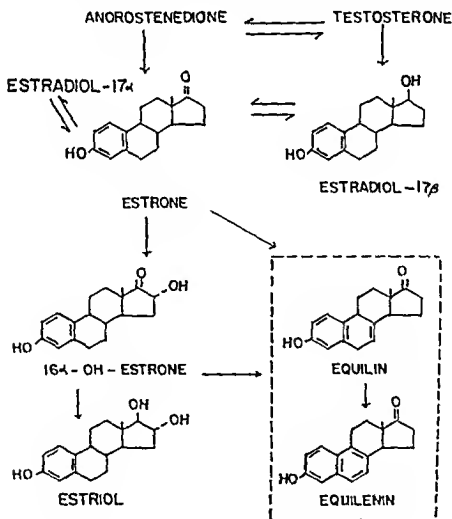
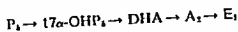


FIG. 6. Metabolism of androgens to estrogens.



Estrogens can arise from the incubation of tissue with acetate, cholesterol, or androgens. Rather than three independent pathways, there is one pathway extending from acetate through cholesterol to  $E_{2-\beta}$  or  $E_1$ . A variation of this pathway exists in the adrenal gland (133):



Total synthesis of  $E_{2-\beta}$  or  $E_1$  from acetate has been demonstrated in the pig ovary (211). Based on this and other studies, it is reasonable to state that acetate is a precursor not only of cholesterol and the neutral steroids, but also of E. Using "FSH stimulated" ovaries, Ryan *et al.* (168) were able to demonstrate the conversion of  $P_4$  to  $E_1$  and  $E_{2-\beta}$ . The link between cholesterol and  $P_4$  is well established. Fieser (66) postulated that T could be converted to E by cleavage of the angular methyl group at C-10. Baggett *et al.* (12) then showed that  $^{14}\text{C-T}$  was converted to  $^{14}\text{C-E}_{2-\beta}$  using human ovarian slices and horse testes. Important findings in this series of studies were the stimulating effect of FSH on the aromatization of T to form  $E_{2-\beta}$  by dog ovary slices (99) and the isolation of an active aromatizing enzyme system in human placental microsomes (169). The addition of NADPH and oxygen caused  $A_2$  to go to  $E_1$  in 50 to 100% yields.

The elucidation of the pathway from A to E began when Meyer observed that 19-OHA<sub>2</sub> was a more active precursor of  $E_1$  than  $A_2$ , indicating it was an intermediate (134). Follicular fluid of cows also converts the 19-OHA<sub>2</sub> to  $E_1$  (135). Proof that 19-OHA<sub>2</sub> was involved in the aromatization of the A ring was obtained (118). The C-19 atom is cleaved as formaldehyde from the 19-oxo compound (34). Thus the aromatization of ring A results from the hydroxylation of C-19 and its removal. Dorfman (59) describes various possibilities by which this transformation takes place. One pathway is shown in Fig. 5.

Heard *et al.* (87) have elucidated the biosynthesis of the "equine estrogens," equilin and equilenin. These structures are shown in Fig. 6.

## E. CATABOLISM OF ESTROGENS

The metabolism of E's in the domestic animal is shown in Fig. 6 (for references see 60). The metabolic pattern is complicated by the fact that there are at least two E's of ovarian origin,  $E_2$  and  $E_1$ , and a number of other E's, among them being  $E_{2-\alpha}$  and  $E_3$ , that have been found in the placenta, blood, urine, and other tissues.  $E_{2-\beta}$  and  $E_1$  are thought to be the secreted forms and the others arise from catabolism.  $E_{2-\beta}$  and  $E_{2-\alpha}$  are interconvertible by a 17-HSD.  $E_1$  occupies the central position.  $E_3$  arises via 16-hydroxylation of  $E_1$  to form 16 $\alpha$ -OHE<sub>1</sub> and a reduction of the ketone group at C-17.  $E_1$  and  $E_{2-\beta}$  can also be hydroxylated at the C-2

and C-6 positions. These compounds have been isolated from human urine (for references, see 35). In the cow, ewe, goat, dog, and rabbit, the metabolic pattern appears to be  $E_{2-\beta}$ ,  $E_1$ , and  $E_{2-\alpha}$  with  $E_{2-\alpha}$  as the major end-product. In the sow,  $E_{2-\alpha}$  is missing;  $E_1$  is the major endproduct.

Unlike the human, the main excretory route for E's in some of the domestic species is the bile and feces.  $E_{2-\beta}$  has been isolated from bovine bile and rises twofold in the feces during pregnancy.  $E_1$  is tenfold less than  $E_2$ . In sheep, the same appears to be true. In the horse, the main route is the urine with ten catabolites being reported, including  $E_1$  and  $E_{2-\beta}$  (207). In ruminants,  $E_{2-\alpha}$  is the major urinary product followed by  $E_1$  (207). The  $E_{2-\alpha}$  has very low biological activity and so is thought to be the end product of E metabolism, analogous to  $E_1$  in man. Mellin and Erb (132) injected  $^{14}\text{C}-E_{2-\beta}$  i.v. into a heifer during the estrous cycle and found that  $E_{2-\alpha}$ ,  $E_{2-\beta}$ , and  $E_1$  comprised, respectively, 81, 3, and 12% of the urinary product recovered with more than two-thirds being excreted during the first 24 hours. The largest peak of total E occurred during the 3 days preceding ovulation. In the pig,  $E_1$  is by far the major urinary metabolite.  $E_1$  is present while  $E_{2-\beta}$  and  $E_{2-\alpha}$  may be present (207). Urinary  $E_1$  rose sharply "between days 20 and 30 of pregnancy, fell for 10 days, then rose steadily until a week before parturition" (31). Recently, Knight *et al.* (133a) using an indwelling cannula in the radial vein have shown that plasma estrogens follow a similar pattern during early pregnancy. Both  $E_1$  and  $E_{2-\beta}$  rose to a peak of 20 pg/ml between days 20 and 30 then fell to a low level by day 40. After day 70 the estrogens gradually rose to a level exceeding 1 ng/ml by day 100. According to three reports (207), the ewe excretes little E in the urine, even during pregnancy.

The liver is considered to be the major site of catabolism of E's as well as other hormones. Zondek (3) reported that the inactivation of estrogens by liver was an enzymatic process rather than being due to conjugation. Schiller and Pincus (178) observed a conversion of  $E_{2-\beta}$  to a less active estrogen by the liver. Hepatectomy diminished the conversion of  $E_1$  to  $E_2$ , but not of  $E_1$  to  $E_{2-\beta}$ . A whole set of NADPH-dependent oxidative enzymes that catabolize E's have been demonstrated in the liver (see 3 for references). Dehydrogenation of  $E_{2-\beta}$  to form  $E_1$  by a protein fraction from the bovine liver has been reported (116).

Cultures of kidney, endometrium, liver, and testes interconvert  $E_1$  and  $E_{2-\beta}$ , thus demonstrating the presence of the appropriate dehydrogenases (133). Red blood cells interconvert  $E_1$  and  $E_{2-\beta}$  in the sheep, horse, pig, and dog (207). In cattle, however, red blood cells interconvert  $E_1$  to  $E_{2-\alpha}$  whereas  $E_{2-\beta}$  is irreversibly transformed to  $E_1$ . Within the last 3-5 years, there has been increasing interest in fetal metabolism of steroid hormones and its involvement in such phenomena as implantation and parturition.

Pig blastocysts collected on day 16 after mating can synthesize free and conjugated E's (156) whereas those collected on day 10 cannot. HSD enzymes which convert A to E have been demonstrated histochemically in blastocysts by day 12 of pregnancy (67).

## 1. Steroidogenic Enzymes

Three types of enzymes are responsible for the synthesis and catabolism of  $P_4$ : (1) dehydrogenases which add to or remove hydrogen from carbons of the steroid nucleus, (2) hydroxylases which add hydroxyl groups to the steroid nucleus, and (3) isomerases which transfer a double bond between carbons after a dehydrogenase has acted on the nucleus. These stereospecific enzymes synthesize and degrade  $P_4$ , A, and E. Hydroxylations occur at the C-20 and C-22 of cholesterol, C-17 of  $P_4$  and  $P_5$ , and C-16 of estrone. Electrons are transported down a multienzyme complex having the components shown in Fig. 7. Electrons are passed from the nonheme iron protein (NHF $FeP$ ) to the  $Fe^{2+}$ -cytochrome P450 protein. The reduced P450 reacts with  $O_2$  and transfers one atom of oxygen to the appropriate site on the steroid. The other atom of oxygen and  $2H^+$  from the medium form water. Thus there are two substrates, both of which are oxidized. The hydroxylase enzyme complex is also called a mixed function oxidase.

## F. CONJUGATES OF PROGESTERONE AND ESTROGEN

Simple steroids which are nonpolar are converted to esters which are more polar. An ester is formed by the reaction of an acid, such as a sulfuric or glucuronic acid, with the hydroxyl group on the steroid. The liver forms sulfate esters of E and  $3\beta$ -hydroxysteroids. Sulfokinases utilize ATP and transfer sulfate from phosphoadenosine phosphosulfate to the steroid. In the liver an enzyme, glucuronosyltransferase, forms glucuronosides by transferring glucuronic acid from uridine diphosphoglucuronic acid to the steroid.

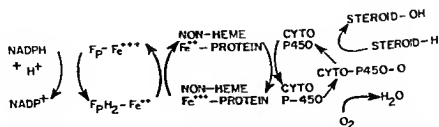


FIG. 7. Hydroxylase or mixed function oxidase system in mitochondria of steroid hormone producing glands.

Conjugation of a hormone should not be regarded as a definite inactivation of the hormone. Certainly conjugation appears to be an essential step prior to excretion. Adlercreutz (3) states, however, that estrogen sulfates may act as primary hormones just as do the unconjugated hormones. The E's are conjugated in one organ and, following hydrolysis in another organ, may exert a biological effect that may be the same or different as that of the free E. In a recent study (165) the incidence of free and conjugated E's in pig plasma during gestation was reported. E<sub>1</sub>-sulfate became detectable in maternal plasma by day 16 of pregnancy and rose to a peak of 3 ng/ml between day 23 and 30, but free E<sub>1</sub> did not appear until day 70, again suggesting, like an earlier report (156), that E<sub>1</sub>-sulfate may be implicated in implantation. The detection of no E<sub>1</sub> during early pregnancy is in contrast to a more recent study (113a).

## G. SECRETION OF PROGESTERONE

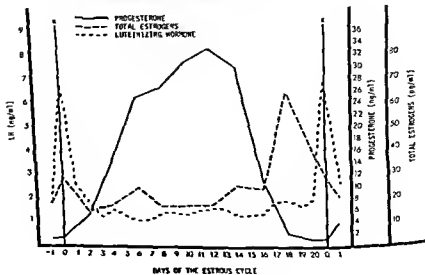
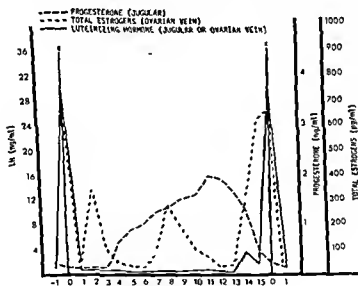
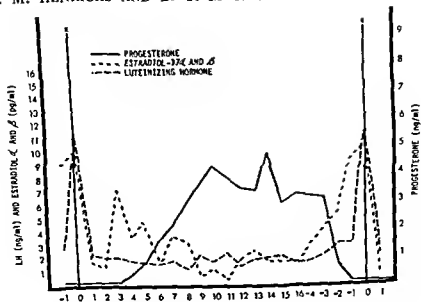
### 1. Sources and Levels in Domestic Animals

P<sub>4</sub> is secreted by the ovary, placenta, and adrenal cortex. The major site of production occurs within the CL, even during the latter part of pregnancy in many species. In the ewe and mare the placenta produces sufficient P<sub>4</sub> after the first one-third and one-half of pregnancy, respectively, to maintain it. In the sow and cow, the ovary is required for the major portion, if not all, of the gestation period (see 40 for a discussion). A daily injection of 28 mg of P<sub>4</sub> maintained pregnancy in ovariectomized gilts (63). It appears that the adrenal gland can contribute some P<sub>4</sub> to the circulating level since a level of 0.8 ng/ml has been measured in ACTH-treated ovariectomized heifers (209).

The concentration of P<sub>4</sub> in the CL parallels the development and regression of the tissue during the cycle. In the cow it ranges from 65 µg/gm on day 12 to 12 µg/gm on day 20 (89). The level of P<sub>4</sub> in peripheral plasma ranges from <1 ng/ml during estrus to a level of 5 to 7 ng/ml between days 10 to 16, after which a rapid decline to a low level occurs, usually <1 ng/ml, followed by estrus (91). The pattern is shown in Fig. 8. The progestin, 20β-OHP, has been identified in the CL, ovary, and adrenal gland.

In the CL of the ewe, P<sub>4</sub> levels are much lower than in the cow (24 µg/gm on day 12) (197). As expected, the levels in plasma (194) are also lower (3 ng/ml on day 12) (see Fig. 8). Another progestin, 20α-OHP, occurs in the ovary and placenta (75).

P<sub>4</sub> concentrations in the CL of the sow range from 47 µg/gm on day 1 after estrus to 69 µg/gm on days 12 to 16, then undergo a decline to 15



$\mu\text{g/gm}$  over the next 3–5 days (127). Henricks *et al.* (93) have reported gonadal hormone and LH levels in pig plasma during the estrous cycle (Fig. 8).  $P_4$  levels are three to four times higher than in the cow—a reflection of the multiple CL in the pig, ranging from 1 to 2 ng/ml on day 2 of the cycle and between 25 and 35 ng/ml on days 12 to 14. Usually levels decrease to one-half the peak level by day 16 and estrus occurs 4–6 days later. A similar profile occurs during the first 14 days of pregnancy (80). Guthrie *et al.* (81) have observed that in superovulated gilts there is good correlation between CL number and  $P_4$  level. As in bovine CL,  $20\beta\text{-OHP}$  has been identified in pig CL (30).

The half-life of  $P_4$  in the cow exhibits a biphasic curve; phase 1 lasting 3 minutes and phase 2 lasting 28 minutes (106). Production rates have been measured in sheep and goats. During the luteal phase of the sheep, the daily output ranges between 4–6 mg (196) and 11–19 mg (201). Of considerable interest is the occurrence of  $P_4$  in milk with 10 to 30 ng/ml being measured (98).

## 2. Control Mechanisms

a. LH. It has been shown by both *in vivo* and *in vitro* methods that gonadotropins stimulate ovarian  $P_4$  synthesis. The addition of LH, HCG, and FSH contaminated with LH to bovine luteal slices stimulated the synthesis of  $P_4$ , whereas prolactin, ACTH, and serum albumin did not. LH acted similarly on CL of the pig, rabbit, and sheep (174).

A hypothesis for the action of ACTH on steroidogenesis in the adrenal gland was proposed by Haynes and Berthet (175) and has received wide acceptance not only as a specific phenomenon of this tropic hormone, but as the means by which LH stimulates steroidogenesis in the CL. The concept states that the tropic hormone acts upon adenyl cyclase in the membrane of the target cell, causing an increase in cAMP, which, in turn activates phosphorylase which increases glucose phosphate for the hexose monophosphate shunt pathway. This pathway increases NADPH, a co-factor necessary for steroidogenesis. Evidence for the applicability of at least a part of this concept to LH action on the CL is summarized by Dorfman (55). Briefly, phosphorylase activity in bovine CL and its stimulation by LH have been reported. The increase in phosphorylase activity

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FIG. 8. Peripheral plasma levels of progesterone, total estrogen ( $E_2 + E_1$ ) and LH in Holstein cows (top), ewes (center), and sows (bottom). LH values are expressed in terms of NIH-LH-B7 (cows as assayed by W. Hansel), NIH-LH-St (ewes as assayed by Scaramuzzi), and LER-786-3 (sows as assayed by Henricks). This figure is reproduced from reference (85) by permission of *American Zoologist*.

and  $P_4$  biosynthesis are correlated. cAMP is present in bovine CL, and its concentration is stimulated many fold, specifically by LH.

Savard (174) has summarized his and others' work and reports the following: exogenous cAMP mimics LH in its effect on steroidogenesis, intact cells are required, the two effects are not additive, and stimulation by LH and cAMP fails in the presence of inhibitors of protein synthesis. LH may be stimulating the synthesis of steroidogenic proteins rather than NADPH. The activity of side chain cleavage enzyme is not increased by previous exposure to LH (174). Indeed, the steroidogenic response to LH is too rapid for an induction of enzyme synthesis to occur. The report of Ungar *et al.* (204) may indicate that a nonenzymic protein isolated from adrenal mitochondria increases the activity of side chain cleavage enzyme by acting as a carrier for cholesterol. It may facilitate the transfer of cholesterol into the mitochondria. The focal point of LH and its "signal transmitter," cAMP, may be generation of such a carrier protein. A second mechanism of action of LH may also be related to availability of cholesterol for  $P_4$  biosynthesis, but at a different level. Behrman and Armstrong (19) administered LH i.v. to rats and showed a significant increase in cholesterol esterase activity within 15 minutes. This enzyme could be converting the ester to free cholesterol to be used for synthesis of  $P_2$ .

b. FSH. Savard (173) summarized his studies of this hormone and reported that FSH preparations, when added to bovine CL slices, were inactive except when contaminated with LH.

c. PROLACTIN. This hormone stimulates  $P_4$  synthesis very little based on *in vitro* studies using bovine CL slices (174) and many other studies using other species. It does not increase phosphorylase activity or cAMP or increase glucose or cholesterol metabolism (8). Prolactin replenishes ovarian cholesterol stores in rabbits (55). Although prolactin appears to be essential to CL function in rodents, it has not been shown to be required for luteotropic support in domestic animals (85).

## H. SECRETION OF ESTROGEN

### 1. Sources and Levels in Domestic Animals

The main sources of E are the ovary and the placenta. Indeed, during the latter stages of pregnancy, the hormone is produced in great quantities in most species. The testes is a significant source of E in a few species such as the stallion and boar. The adrenal gland is a minor source (208).

The ovarian unit responsible for secretion is the follicle. The follicular

fluid, in general, is a very rich source of hormonal steroids and their precursors. In the cow  $E_{2-\beta}$  and  $E_1$  predominate, while in the mare it is  $E_{2-\beta}$  and  $6\alpha\text{-OH-}E_{2-\beta}$  with some  $E_1$  present (195). The thecal cells of the follicle have a greater activity of the 17-hydroxylase and desmolase than granulosa cells and are thought to be the main source of E during the follicular phase (189).

Production rates of E have been measured in the ewe and goat. Maximum secretory rates of 6 to 9  $\mu\text{g}/24$  hours of  $E_{2-\beta}$  have been reported (13, 48) for the nonpregnant sheep. In pregnant sheep, 1.8 mg of  $E_1$  and 28 mg of  $E_{2-\beta}$  have been reported (208). Production rates (milligrams per day) can only be estimated for the cow on the basis of studies on urinary and fecal excretion (208). Hundreds of milligrams are probably produced during late pregnancy.

E levels in the plasma of nonpregnant domestic animals are low relative to the levels found in rodents and primates. Peak values occur at or just prior to estrus and range from 12 to 20  $\mu\text{g}/\text{ml}$  in the cow to 50 to 70  $\mu\text{g}/\text{ml}$  in the pig. Figure 8 shows the E profiles for the cow, ewe, and sow during the estrous cycle. During pregnancy, the levels of free and conjugated estrogen reach levels of nanograms per milliliter just prior to parturition. In both the cow (159) and the pig (165), plasma levels of estrogen sulfates were considerably higher than free estrogens.

Of the two active E's,  $E_{2-\beta}$  and  $E_1$ , the former predominates slightly in the blood of the cow. In a study of 12 nonpregnant heifers, the ratio of unconjugated  $E_{2-\beta}$  to  $E_1$  ranged from 3:1 to 1:1 depending on the stage of the estrous cycle (Henricks, unpublished data). The wide ratio occurred at estrus. In addition to  $E_{2-\beta}$  and  $E_1$ ,  $E_{2-\alpha}$  is present in sheep and cow blood (53, 166). Our understanding of the dynamics of E secretion is based on peripheral plasma levels. Data on ovarian-venous (O-V) levels of hormones in domestic animals are now becoming available. A brief study (146) indicates that in O-V plasma,  $E_{2-\beta}$  is 50–200 times higher than in peripheral blood and  $E_1$  is 20–100 times higher. Sampling at 2-hour intervals showed pulses of  $E_{2-\beta}$  occurring during the 2 days before estrus and between days 3 and 6 of the cycle. Intensive studies of this type are needed to describe the actual plasma profiles of E and to elucidate their role in reproductive processes.

E is present in the milk but in very low amounts based on bioassay. Lunaas (119) has detected  $E_{2-\beta}$  and  $E_1$ . Colostrum contains quantities ranging up to several nanograms per milliliter of both free and conjugated E's. The amount of free E in milk reflects the amount in the plasma during the cycle (142). The amount in milk is three to four times higher than that in peripheral plasma, which may indicate an active uptake by the mammary gland.



## 2. Control Mechanisms

It is reasonable to assume that the gonadotropins play a role in the control of secretion, but our understanding of this control is nil. FSH has been shown to increase the conversion of T to  $E_{2-\beta}$  by dog ovaries as does LH and HCG (99). With cow (153) or pig (160) ovaries, LH was not stimulatory. All of these were *in vitro* studies. Another approach is the infusion of ovaries with gonadotropins and measuring E levels in O-V blood. Both FSH and LH stimulated the conversion of  $P_5$  to  $E_{2-\beta}$  in dogs (1), but not in horses (187). No effect by either hormone was detected in sheep (190). These findings may not be physiologically meaningful. Dobson and Fitzpatrick (54) have shown that in cows the peak in plasma  $E_{2-\beta}$  is attenuated by a single injection of HCG after E began to rise. In addition, LH caused a decline in E secretion by cultured sheep follicles (131). Studies using hypophysectomized animals may be more meaningful as the effects of endogenous gonadotropins are eliminated. To date the rat has been the best species for these studies. Here FSH, treated to reduce LH contamination, induces E secretion (157).

Steinmetz suggests that normally gonadotropins have no direct role in control of ovarian E secretion (195). Rather they exert their effects indirectly by increasing blood flow and stimulating follicular growth. The results from several reports in which granulosa cells were cultured (41, 42) suggest that exposure of the cells to gonadotropins programs the nucleic acids in such a manner as to determine the pattern of steroid synthesis. Finally the selective effect of gonadotropins on follicular growth remains an enigma.

## III. Physiological Effects of Androgens, Progesterone, and Estrogens

### A. INTRODUCTION

Tissues that are acutely responsive to androgen in the male or estrogen and progesterone in the female under normal conditions are termed "target organs." The main organs are the accessory sex organs in the male, the vagina, uterus and mammary gland in the female, pituitary gland, and certain areas of the brain. The physiological effects of the sex hormones can be divided into *gross effects*, such as growth and increased vascularity of an organ, and *specific effects*, such as the effects on metabolites, enzyme systems, and the nervous system. The effects on the nervous system result in characteristic patterns of behavior.

## B. ANDROGENS

The main physiological effects of A in both the gross sense and specific sense, are outlined by Dorfman and Shipley (56) and by Emmens (64). Recent research has dealt with the activity of A at the cellular level. As a result, it is evident that the reproductive biologist must add the word "hormone receptor" to his vocabulary. A brief discussion follows of the specific effects of A on the testes, accessory organs, and behaviorism in the male.

### 1. Testes

The administration of [1,2-<sup>3</sup>H]T to hypophysectomized rats resulted in the binding of T and H<sub>2</sub>T to specific cytoplasmic receptors in the cytoplasmic fraction of seminiferous tubules (86). The cytoplasmic receptor in the tubules was clearly different from the testicular A-binding protein, but similar to the cytoplasmic receptors of epididymis and prostate. These receptors in the tubules may be mediators of the androgenic stimulus to the germ cells.

### 2. Epididymis

Evidence exists for the presence of a H<sub>2</sub>T-binding protein in the cytoplasm of the epididymis (16). The binding protein was organ-specific and possessed a limited capacity to bind A. The binding protein in epididymis differed in its physiochemical properties from those of the binding protein in the ventral prostate and seminal vesicles. H<sub>2</sub>T appears to be the primary A involved in the maintenance of male accessory sex organs (16). Earlier investigators have indicated that the androgenic requirements of accessory organs for normal functioning may differ. It is interesting to speculate that differences in the characteristics of the androgen receptors may play a role in the relative androgen requirements of these organs.

### 3. The Brain and Behaviorism

A higher concentration of H<sub>2</sub>T was found in the brain than in the blood in functionally hepatectomized male rats (184). Both H<sub>2</sub>T and 5 $\alpha$ -androstane-3,17 $\beta$ -diol were present in higher concentrations in the brain than in the blood of totally castrated groups. This suggests that under this *in vivo* condition, rat brain is capable of converting T to androgenic metabolites.

When  $^3\text{H-T}$  and  $^3\text{H-H}_2\text{T}$  were administered to castrated male rats, the  $^3\text{H-T}$  yielded T,  $\text{H}_2\text{T}$ , and  $\text{A}_2$  and the  $^3\text{H-H}_2\text{T}$  resulted in the presence of  $\text{H}_2\text{T}$ , androstenediol, and androstenediol in brain tissue attesting to the capability of brain tissue to metabolize administered A.

Although  $\text{H}_2\text{T}$  has been suggested as the primary androgen in the maintenance of male accessory sex organs, it does not appear to be effective in the maintenance of mating behavior in castrated male rats or to support aggressive behavior in mice (120). All these androgenic effects can be induced with T as the exogenous hormone (213). Readers interested in sexual behavior are referred to the new book by Sandler and Gessa (172).

## C. PROGESTERONE AND ESTROGEN

### 1. Uterus

a. EFFECTS OF PROGESTERONE. The dependence of the uterus on progesterone for its function was pointed out by Corner and Allen (46) in 1929 in their studies of the pig. They stated, "The extracts of corpus luteum contain a special hormone which has as one of its functions the preparation of the uterus for reception of the embryos by inducing progestational proliferation of the endometrium." Studies using mainly the laboratory species have demonstrated that after a period of estrogen stimulation the uterus will proliferate in the presence of  $\text{P}_4$ . Although growth occurs, mitosis is not prominent. The tubular glands undergo changes in length and tortuosity (64), and epithelial cells change form and secrete glycogen (102). The growth-promoting activity does not equal that of  $\text{E}_{2-\beta}$ . During pregnancy, the uterus increases markedly in size and involutes after parturition. The administration of  $\text{P}_4$  to ovariectomized and estrogen-treated animals increases the activity of procollagen hydroxylase, thus promoting the synthesis of collagen (110). Collagenase activity is increased slightly by  $\text{E}_{2-\beta}$  and significantly decreased by  $\text{P}_4$  (44). These reports suggest that  $\text{P}_4$  may be required for myometrial hypertrophy, and after parturition its withdrawal facilitates uterine involution.

Csapo (49) has proposed that during pregnancy  $\text{P}_4$  is the factor responsible for myometrial quiescence. Uterine responsiveness to E and oxytocin disappears.  $\text{P}_4$  increases the membrane potential and stimulus required to induce contraction of the myometrial tissue. When the  $\text{P}_4$  "block" is in effect, electrical activity is suppressed. Uterine levels of  $\text{P}_4$  correlate more closely with the maintenance of pregnancy than do plasma levels (50). In early studies, isotopic  $\text{P}_4$  could not be localized in the uterus (24), perhaps because it had been converted to polar metabolites. More recently, Wiest and Rao (217) reported that uterine  $\text{P}_4$  exceeded

that in plasma, whereas uterine  $20\alpha$ -OHP was not greater. Although a specific  $P_4$ -binding protein is present in the endometrial cytosol, it remains to be determined if the protein is a receptor. The molecular basis of these phenomena is being studied.

$P_4$  regulates the activities of endometrial enzymes. It increases carbonic anhydrase activity (121), although this event may not be necessary for the hormone to induce the typical tissue morphology (115). The hormone increases glucose-6-phosphate dehydrogenase, isocitric dehydrogenase, and NADP-malic enzyme as well as total uterine RNA and DNA content (117). Leucine aminopeptidase is largely controlled by  $P_4$  (20). While it increases phosphatase activity, it antagonizes the greater response given by  $E_2$  (145).  $E_2$  induces pyruvate kinase activity, whereas  $P_4$  inhibits the response (51). Thus  $P_4$  can either increase enzyme activity or act antagonistically to the action of  $E$ .

**b. EFFECTS OF ESTROGEN.** In the uterus,  $E$  acts as a growth hormone. Following a single injection of  $E$  into a spayed rat, the uterus undergoes hyperemia within 4 hours (118), an increase in water content within 6 hours, and an increase in dry matter content within 30 hours (10). The first morphological sign of stimulation is the division of cells in the endometrial epithelium and glands, followed by a secretion into the uterine lumen and a ballooning of the uterus.

In the ovariectomized cow,  $E$  increases the height of the uterine epithelium, size of the glands, and stromal edema (9). Glycogen content of the epithelium is reduced, and endometrial arterioles are maintained (84).

$E$  also influences the myometrium to undergo growth. Hypertrophy of both the circular and longitudinal muscles occurs. Uterine contractility is under  $E$  control, although  $P_4$  also plays an essential role. Contractions occur at a low level during the luteal phase, gradually increase in frequency and amplitude during the follicular phase, and culminate at estrus. This has been shown for a number of species, e.g., rabbit, sow, and cow. Uterine motility increases in the ovariectomized cow upon injection of  $E$  (8).

In the uterus of the spayed mouse, changes in the appearance of the luminal cell surface and the plasma membrane occur within 4 hours after i.v. administration of  $E_2$ . By 20 hours, cell growth had occurred. Other than the early uptake of water, no other morphological change has been described that is indicative of the early action of  $E$ . Later changes then are secondary to more fundamental changes caused by  $E$ .

Respiration of uterine segments increased within 1 hour of injection of  $E_2$  into the spayed rat (199). Phospholipid (PL) content increased within 1 hour (144). This was followed by a rise in RNA by 6 hours, and

increased further by 24 hours. Protein synthesis did not rise until after 12 hours. Capacity to synthesize PL increases.  $E_{2-\beta}$ ,  $E_1$ , and  $E_3$  stimulated incorporation of orthophosphate into PL's, but DES did not, nor did  $P_4$  nor cortisol (4).  $E_{2-\beta}$  is involved in the reactions leading to synthesis of nucleotides. Incorporation of  $^{14}CO_2$  into guanine nucleotides was significantly stimulated within 1 hour of E administration (144). From work on enzymes which activate amino acids and accelerate protein synthesis, Mueller (144) concluded that early metabolic changes are geared to the levels of rate-limiting enzymes and that E acts only by stimulating *de novo* synthesis of these enzymes. Puromycin, an inhibitor of protein synthesis, prevents incorporation of glycine into protein, but not into RNA or incorporation of  $P_i$  into phosphatides. It is interesting to note then that the inhibitor prevented all of the early E responses (152). It was concluded that E is concerned with the stimulation of protein synthesis. Another inhibitor, actinomycin, blocks the synthesis of RNA. It also prevented the increase in PL and protein synthesis in the uterus caused by E, thus suggesting that RNA synthesis is a prerequisite for the greater protein synthesis due to E (203). Notebloom and Gorski (147) have concluded that E induces the synthesis of RNA polymerase which then leads to accelerated RNA synthesis and hence to a synthesis of the enzymes involved in metabolism of nutrients. Indeed, recent efforts have centered on proteins synthesized soon after  $E_{2-\beta}$  administration.

As mentioned earlier, hyperemia, increased capillary permeability, and release of membrane-bound materials such as histamine (198) are also significant events in the action of E on the uterus. These phenomena would allow more metabolites to arrive at the site of growth. Thus E may be acting in the uterus at both the cell membrane and the chromatin to bring about its specific and powerful effects on this target organ.

This discussion has dealt with the biochemical actions of E which, of necessity, overlaps into a discussion of mechanism of action. For a discussion of uptake of E and  $P_4$ , binding to the tissue receptors, and c-AMP involvement see Chapter 6.

## 2. The Oviduct and Mammary Gland

In the rabbit,  $P_4$  appears to repress oviducal contraction while E is stimulatory (104, 212). Other studies (27, 28), on the other hand, indicate that the oviducal activity increases near ovulation due to withdrawal of E and secretion of  $P_4$  from the follicles. Ovariectomy reduces rate and amplitude of contractions. In any case, the ratio of  $P_4$  to E would seem to be important in the retention of the ovum in the oviduct for the optimum time period. As E dosage increased in castrated rabbits, more ova

were found in the oviducts and fewer in the uterine (148). Two studies (25, 77) have shown that  $P_4$  accelerates ova transport in rabbits. Hafez *et al.* (82) reported the same results in superovulated cows.

Recent studies (for two reviews see 17, 21) have focused on the flow of oviducal and uterine fluids and their protein composition.  $E_{2-\beta}$  gave rise to serum-identical proteins and suppressed uterine-specific proteins.  $P_4$  had the opposite effect (21). A uterine-specific protein, "uteroglobin," has been partially characterized and may be identical to "blastokinin" (114).

Glucose, maltose, and fructose are present in oviducal extracts of rabbits (79).  $E$  increases the oligosaccharide level, whereas  $P_4$  decreases it. Glucose, lactate, and pyruvate levels increase after ovulation and may stimulate metabolism of the ovum (100).  $P_4$  injection in the rabbit doubled inositol content of oviducal fluid (78). It decreased fluid pH and increased carbonic anhydrase activity in both rabbits and cows (121). In the cow, at estrus, fluid  $Na^+$  and  $K^+$  concentrations were lowest (151). In the ewe and cow (155), the formation of oviducal fluid was greatest around estrus and diminished during the luteal phase with large increases in protein and glucose content occurring at estrus.  $E$  would appear to be involved in these changes.

A review of the effects of gonadal hormones on mammary gland growth and secretion is provided in Chapter 14. Turner and Cowie have studied independently the effects of estrogen and progestins on the mammary glands of cows and goats (23, 47, 162, 202).

### 3. Pituitary Gland and Ovary

The presence of a functional corpus luteum is associated with both the lack of ovulation and estrus. Daily injections of 10 mg of  $P_4$  in the ewe and 50–100 mg in the cow and pig during the estrous cycle have the same effect (for references see Foote, 154). Cows return to estrus 4–7 days after the end of treatment. It has been shown that  $E$  preceded by  $P_4$  are required by the ewe for normal estrus (68).  $P_4$  may have a conditioning effect. A preovulatory peak of  $P_4$  has been reported in the cow (11). Others have not detected such a peak.  $E_{2-\beta}$  and  $P_4$  together gave a more normal estrus than  $E_{2-\beta}$  alone.

The general concept that  $P_4$  suppresses LH secretion via a negative feedback mechanism, at least insofar as cyclic release of preovulatory amounts of LH is concerned, is widely accepted. A number of workers have shown that in the cow no significant rise in plasma LH occurs until plasma  $P_4$  levels decline to less than 1 ng/ml (see Fig. 8). In the presence of high  $P_4$  levels, however, low levels of LH are maintained that are suf-

ficient to maintain a functional corpus luteum. Thus the control of LH secretion by  $P_4$  may involve two "centers."

Plasma E levels associated with the ovulatory follicle do not begin to rise above a base line level during the follicular phase of the cycle until plasma  $P_4$  begins to decline. The inverse relationship between the levels of the two hormones can be seen for all three species in Fig. 8. In the cow, Henrieks *et al.* (92) reported that plasma E rose on the day after  $P_4$  had fallen significantly and peaks about 1 day before estrus. It seems well-established that the estrogen surge causes LH release in the cow, ewe, and sow. Piper and Foote (158) demonstrated that  $E_{2-\beta}$  induced ovulation in the ewe when given on day 4 of the cycle. Goding *et al.* (73) gave  $E_{2-\beta}$  to anestrus sheep and found an LH peak 8–12 hours later. The interval between  $E_{2-\beta}$  injection and peak release of LH decreased in the ewe as the dose increased (161). In a study of the cow (94), plasma E levels were dose related to PMSG, and as E level rose the interval from onset of estrus to peak LH levels decreased. An LH surge in the ovariectomized cow occurred 16–28 hours after an im injection of  $E_{2-\beta}$  (101).

The occurrence of an LH surge in response to  $E_{2-\beta}$  administration probably depends on the level of  $P_4$  present.  $E_{2-\beta}$  did not evoke a surge in the ewe on day 10 of the cycle, but did on day 3 (29). There appears to be a definite inhibition of the naturally occurring or estrogen-induced LH surge by  $P_4$  administration in the ewe (70).

Reeves *et al.* (162) have shown that LH release in response to GnRH in ewes was greatest on day 1 of the cycle relative to any other time tested. Thus pituitary sensitivity to GnRH may be enhanced by  $P_4$  removal or by E stimulation. In beef heifers administered GnRH on days 0, 16, 18, or 20 of the estrous cycle, increases in serum LH were observed on all days with the greatest response occurring on the day of estrus (109). A relatively small response was measured on day 16, a time when  $P_4$  levels are high. The hormones may be affecting LHRF release, or sensitizing the pituitary cells to the local level of LHRF, or having a direct effect on the pituitary cell's release of LH. Evidence for pituitary sensitization comes from experiments with diestrous rats (7) and anestrus ewes (163). The LH level was significantly higher in animals pretreated with  $E_{2-\beta}$  and LHRF than in those treated with LHRF alone. In this setting,  $P_4$  may be preventing the ovulatory surge of LH by acting at the hypothalamic level to alter LHRF release or at the pituitary level by affecting sensitization.

$P_4$  and E influence luteal function.  $P_4$  acts to hasten CL regression in the nonpregnant ewe and cow, but not in the nonpregnant sow. In pregnant sows,  $P_4$  injection reduced CL weight, but not  $P_4$  concentration. Exogenous  $E_{2-\beta}$  causes CL regression in cattle, however, this phenom-

enon can be blocked by administering gonadotropins. Estrogens also induce CL regression in cycling, pregnant, and hysterectomized heifers. In sheep,  $E_{2-\beta}$  is luteolytic during the first half of the cycle. In the non-gravid pig, exogenous estrogen is luteotropic (68).

#### IV. Uterine Factors

##### A. UTERINE LUTEOLYTIC FACTOR (ULF)

In a number of species, including the cow, ewe, sow, and mare, the uterus plays an important role in the regulation of the function of the corpus luteum (6, 26). In the guinea pig, pig, sheep and cow, the CL remains functional for a period equivalent to the length of pregnancy if the uterus is removed. It appears that the non-gravid uterus is required for regression of the corpus luteum. Further work has demonstrated that removal of one uterine horn in these species leads to regression of CL in the ovary adjacent to the remaining non-gravid horn and maintenance of the CL contralateral to the horn (71). It appears that the uterus in these species exerts a local luteolytic action that is, at least partially, systemic in nature. If this is true, it would seem possible to isolate the factor(s) that is responsible for luteolysis. Uterine flushings from the pig on days 12–18 of the cycle degrade granulosa cells in a monolayer system as reported by Schomberg (179). Luteal regression did not occur when the flushings were infused into pigs. Chemical studies of the uterine flushings indicated that the factor(s) was nondialyzable, heat-labile, and of high molecular weight—the properties attributable to a protein (179). Similar studies have been done using endometrial extracts from non-gravid uteri of sheep with somewhat less convincing results (39).

Prostaglandin  $F_{2\alpha}$  has been suggested to be the ULF (74). Exogenous  $PGF_{2\alpha}$  is luteolytic in the ewe (74) and the cow (167), and there is evidence for a preferential transfer of PGF infused into the uterine ovarian vein to the ovarian artery entwined about the vein of the ewe (74). The infusion of 20 to 25  $\mu\text{g}/\text{hour}$  of  $PGF_{2\alpha}$  for 4 to 8 hours into the uterine vein adjacent to the CL is sufficient to cause luteolysis in the ewe (130). A similar transfer of  $PGF_{2\alpha}$  deposited into the uterine lumen to the ovarian artery has been demonstrated in the cow (96); however, an elevated level of  $PGF_{2\alpha}$  in the ovarian artery of the normal cow at any stage of the estrous cycle has not been demonstrated (181). It should be noted that the bioassay of various fractions of uterine endometrium for luteolytic activity indicated the active principle was arachidonic acid, a precursor of  $PGF_{2\alpha}$  (182).



Increased plasma estrogen levels occurred following the administration of  $\text{PGF}_{2\alpha}$  i.v. (96), as well as when it was injected directly into the CL (95). Plasma  $\text{P}_4$  levels decreased significantly from 5.5 to about 2.5 ng/ml. These findings and the report (97) that doses of  $\text{PGF}_{2\alpha}$  that cause complete luteolysis in the ewe, fail to do so after X-irradiation of ovaries to destroy the follicles, lead to the suggestion that estrogen may be involved in the luteolytic process in the ewe and cow.

## B. UTERINE PROTEINS ASSOCIATED WITH PREGNANCY

Specific proteins have been found in uterine secretions of pregnant mammals at times that are coincident with certain stages of embryonic development. This has led to the hypothesis that these proteins are required for the growth or differentiation of the embryo. One especially prominent protein among the six fractions identifiable upon separation of rabbit flushings on Sephadex G200 chromatography is "blastokinin," so named by Krishnan and Daniel (114). They suggested that blastokinin was necessary for inducing and regulating blastocyst formation. Beier (21) isolated a similar fraction and named it "uteroglobin." It migrated in the same region upon gel electrophoresis (postalbumin region) and had a similar effect on the morula stage of the embryo. It now appears that the two proteins are identical and they exert their effect on growth rather than blastocyst formation. The protein attains a maximum concentration on the fifth postovulatory day in the rabbit and remains high until day 9 when it begins to decrease (22). It stimulates the synthesis of protein (108) and RNA (205) in blastocysts. The protein is present in ovariectomized animals receiving  $\text{P}_4$  or  $\text{P}_4 + \text{E}_2 \beta$  and absent in animals treated with  $\text{E}_2 \beta$  alone. The administration of  $\text{P}_4$  to nonpregnant, estrous animals for 5 days induces the same level of blastokinin as found in flushings from rabbits on day 5 of pregnancy (128), thus suggesting the synthesis of this protein is  $\text{P}_4$ -dependent.

On the basis of several lines of evidence (17) it appears that  $\text{P}_4$  is the primary hormone regulating synthesis and/or secretion of uterine-specific proteins in the pig. In addition to an increase in total protein content of a single uterine flushing until day 15 of the estrous cycle, then decreasing sharply, two protein fractions were present only during the luteal phase. Three other fractions were present throughout the cycle. A specific protein, purple intrauterine glycoprotein, has been isolated from uterine flushings of gilts between days 12 and 16 of the cycle. It has been shown to have a molecular weight of 32,000, 12% carbohydrate, one atom of iron per molecule, and a high alkaline phosphatase activity. The site of synthesis is the epithelial cells of the uterine surface and glands. It collects

on the stroma to be absorbed via the placental areolae and is sequestered in the allantoic fluid. Passive immunization of gilts against the purple protein resulted in a reduction in placental size but not embryonic development.

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# 5 Biological and Immunological Assay of Gonadotropic and Gonadal Hormones

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## I. Introduction

In order to obtain a complete understanding of the complex endocrinological events required for normal reproductive function, quantification of the reproductive hormones in various tissues and fluids is necessary. Historically, a variety of assays have been developed for this purpose. The first assays took advantage of the ability of the hormone to be measured to produce an unique biological effect in test animals. In general, the

quantitative aspects of these assays were based on increased weight of target organs or histological changes in the tissues of target organs. By present standards these assays were imprecise, expensive, tedious, and insensitive. However, data obtained using these procedures have generally been proved correct and provide the foundations of modern endocrinology.

The biological assays were subsequently replaced by direct chemical procedures for the quantitative measurement of steroid hormones. In general, the chemical assays were quite specific and relatively sensitive, but were tedious since extensive sample preparation was required prior to final quantification. In addition, most chemical assays did not have sufficient sensitivity to allow the quantification of steroid hormones in small amounts of serum or plasma. In recent years there have been numerous improvements made in the methods used for the quantification of hormones. The improvements are primarily the result of the introduction of radioimmunological (5) and competitive protein-binding (46) techniques for hormone analysis. These methodologies provide sufficient sensitivity to measure concentrations of most reproductive hormones in the peripheral circulation and yet were relatively inexpensive and easy to perform. Radioimmunoassays were first developed for relatively large molecular weight, proteinaceous hormones. However, this technique has subsequently been adapted for use in measurement of a variety of low molecular weight substances.

## II. Bioassay Techniques

A wide variety of bioassays were originally developed for quantitative and qualitative identification of gonadotropic and gonadal hormones. The bioassays described on the following pages are the assays used most widely at the present time. Therefore, presumably they are the simplest and/or most reliable.

### A. ESTROGENS

Bioassays for estrogens include the uterine weight assay and the vaginal smear assay. Both assays are dependent upon changes in target organs induced by estrogens and are performed in ovariectomized rats and mice. The uterine weight assay is based upon the ability of estrogens to increase the weight (uterotropic effect) of the uterus (2, 3). Best results are obtained when the assay is performed using animals that have been ovariectomized for 2 weeks or longer. In general, a dose-response curve is obtained by administering daily subcutaneous injections of estrogens

(1–20 ng/day) to groups of 10 mice for a period of 7 days. Test samples to be assayed are injected at two dose levels into additional groups of animals following the same regimen. On the eighth day the uteri are removed, trimmed of fat, and weighed. The average weight of the uteri from each group of rats is plotted against the dose of estradiol administered to produce a standard dose-response curve. The response obtained in the rats which received the test substance can be quantified by comparison to the standard dose-response curve. The response obtained to the different forms of estrogens is quite variable in this bioassay, with estradiol-17 $\beta$  being the most, and estriol the least, potent (see Chapter 6 for explanation).

A vaginal smear bioassay (58) is based upon the ability of estrogens to promote cornification of the vaginal epithelium in rats or mice (33). At least 3 weeks following castration the animals are divided into groups of ten and given three subcutaneous injections of estrogen in oil at 12-hour intervals. Standard estradiol-17 $\beta$  is injected at dosages ranging from 1.5 to 15 ng and test samples are injected at two dilutions. Vaginal smears are taken at 12-hour intervals beginning 24 hours after the last injection to determine if cornified epithelium is present. A standard dose-response curve is constructed by plotting the percentage of animals in each group which exhibited cornified vaginal smears against the dose of estradiol that each group received. The percentage "positive" smears in the animals given the unknown solution is compared to percentage "positive" smears in animals treated with standard. There is considerable variation in the response of this assay to the various forms of estrogens, with estradiol-17 $\beta$  being the most potent and estriol having the least potency. Both the uterine weight assay and the vaginal smear assay can be influenced greatly by the choice of solvent in which the estrogens are injected (44).

## B. PROGESTERONE

The more commonly used bioassays for progesterone are the decidual cell response in rats (or mice) and the uterine carbonic anhydrase assay in rabbits. These bioassays are effective for the quantification of progesterone, but are not responsive to other naturally occurring progestins.

The decidual cell response assay is most commonly performed in ovariectomized rats that have been primed with estrone to augment the progestational response (4). Groups of rats that have been ovariectomized for 1 week are given 1  $\mu$ g of estrone and the test dosage of progesterone for 5 days. On the fifth day of treatment the rats are laparotomized and one uterine horn is exposed for traumatization (14). The untraumatized horn serves as a control. The incision is closed and the progesterone treatment continues for 4 days. The rats are killed 24 hours after the last injection.

tion, the uterus is removed, trimmed of fat, and each horn is weighed. The increase in weight of the traumatized horn compared to the control horn is proportional to the dosage of progesterone administered. The smallest quantity of progesterone detectable by this assay is in the range of 0.25 mg. A major disadvantage of the decidual cell response assay is that it is extremely sensitive to estrogen (63). Therefore, this assay is not suitable for samples with estrogen contamination or for synthetic progestins with inherent estrogenic activity.

A second relatively common bioassay for progesterone is the uterine carbonic anhydrase assay (51). This assay is based upon the observation that the concentration of carbonic anhydrase in the rabbit uterus is dependent upon progesterone. The concentration of carbonic anhydrase in the uterus may be determined by measuring the production of carbon dioxide by homogenates of endometrial tissue (48). This assay is most sensitive when conducted using endometrial tissue from immature rabbits that have been primed with 5.0  $\mu$ g estradiol-17 $\beta$  daily for 6 days. The standard doses of progesterone and the test substances are administered daily for 5 days by subcutaneous injection. The minimum detectable quantity of progesterone in this assay system is 0.1 mg (0.02 mg/day).

### C. ANDROGENS

By far the most common bioassay for androgens is measurement of weight changes of the ventral prostate gland and seminal vesicles in immature or castrated rats or mice. A second, relatively common, assay is measurement of comb weight in immature cockerels that have been treated with androgens. Although these assays have been used to measure the androgenic potency of a variety of steroids, testosterone is the most potent of the naturally occurring androgens in both assay systems.

The ventral prostate/seminal vesicle weight bioassay can be performed in either immature or castrated male rats or mice (17). A period of 2 weeks after castration must be allowed to ensure complete involution of the accessory glands if castrated animals are to be used. Seven subcutaneous injections of the standard doses of testosterone or the test substance are given at daily intervals. Twenty-four hours after the last injection the animals are sacrificed and the ventral lobe of the prostate gland and the seminal vesicles are removed, trimmed of fat, and weighed. Results are expressed in terms of gland weights or as a percentage of body weight. The ventral prostate/seminal vesicle weight assay can detect approximately 0.5 mg testosterone.

The major advantage of the chick comb method for bioassaying androgens is its simplicity (50). The assay is conducted utilizing immature

cockerels 2-3 days of age. Substances to be tested are dissolved in diethyl ether and 0.2 ml of the solution are applied to the lateral surface of the comb at daily intervals for 7 days. Twenty-four hours after the last treatment the birds are sacrificed and the comb is removed and weighed. The data can be expressed as total comb weight or as a percentage of body weight. The increase in comb weight is directly proportional to the dosage of androgen administered. A significant increase in the weight of the comb can be achieved with as little as 10  $\mu$ g of testosterone.

#### D. FOLLICLE-STIMULATING HORMONE (FSH)

Classically, FSH has been assayed by the mouse uterine weight assay or the HCG augmentation assay. Recently, induction of premature ovulation in hamsters has also been used for the bioassay of several gonadotropic hormones. The uterine weight assay for FSH (27) is an indirect assay since it is based upon water imbibition by the uterus as a result of estrogen secretion from ovarian follicles which were stimulated by the FSH (see the uterine weight bioassay for estrogen). Immature female mice (21 days of age) are given subcutaneous injections of FSH (or test substance) at 12-hour intervals for 3 days. The animals are killed 24 hours after the last injection, uteri are removed, trimmed of extraneous tissue, and split down the center of each uterine horn to remove intrauterine water. The tissue is weighed and the mean weight in each group is plotted against the dose of FSH standard used. The results obtained with the test substances are compared to the standard dose-response curve. Since luteinizing hormone (LH) is known to act synergistically with FSH to stimulate secretion of estrogen, contamination of unknowns with LH may result in false high estimates of FSH.

The use of large quantities of HCG to augment responses to FSH has resulted in a decrease in variation due to LH contamination and has increased the sensitivity of the assays to FSH (57). Immature rats or mice (21 days of age) can be used for this assay. The quantitative basis for this assay is an increase in ovarian weight due to FSH stimulation of ovarian follicular growth. The test material is injected with 20 IU HCG divided equally for nine subcutaneous injections (0.5 ml each) administered at 8-hour intervals. The animals are killed 8 hours after the last injection, the ovaries are collected, the bursa is removed, and the ovaries are weighed. Ovarian weight may be increased with 50  $\mu$ g NIH-FSH-S1 administered over the nine injections. The use of excess HCG eliminates variation due to LH contamination of the sample, and thus this has been the most widely used bioassay for FSH in recent years.

Recently, Yang and Papkoff (62) have described a bioassay for FSH

which utilizes premature ovulation in hamsters as the end point. This assay system is affected by other gonadotropic hormones but is simpler and less expensive than other bioassays for FSH. The test material is injected intravenously at 5 AM on the morning of proestrus (12 hours prior to the surge in gonadotropins). The animals are killed at 11 PM of the same day and ovaries with attached oviduct are removed. The cumulus mass is flushed from the oviduct and ova are counted under a dissecting microscope. The number of ova present is related to the dose of FSH injected. This assay can detect 250  $\mu$ g of NIH-FSH-S1.

#### E. LUTEINIZING HORMONE (LH)

The ventral prostate weight assay for LH is an indirect assay based upon the ability of testosterone to stimulate increases in the weight of the accessory sex organs (see the ventral prostate/seminal vesicle weight bioassay for testosterone). LH stimulates the synthesis and secretion of testosterone by the Leydig cells of the testis (21). Best results are obtained in this assay when using hypophysectomized, immature rats, but the assay may be performed in intact, immature rats as well. The substance to be tested is injected subcutaneously, once daily for 4 days. The animals are killed 24 hours after the last injection and the ventral lobe of the prostate gland is removed and weighed. The increase in weight of the prostate gland is proportional to the dosage of LH injected. A significant increase in the weight of the ventral prostate is elicited by 10  $\mu$ g NIH-LH-S1. This assay has been widely used since it does not appear to be affected by FSH contamination of the test material and is relatively easy to perform.

The ovarian ascorbic acid depletion (OAAD) assay has been the most widely used bioassay for LH in recent years. It was developed by Karg (26) and Parlow (49) and is based on the ability of LH to deplete ascorbic acid from corpora lutea of immature pseudopregnant rats. The physiological mechanism involved in this depletion is still not understood. Best results are obtained when the assay is performed on day 7 of pseudopregnancy, but rats between days 5-9 of pseudopregnancy can also be used. One ovary is removed immediately prior to intravenous injection of the test material. The second ovary is removed 3 hours later. The concentration of ascorbic acid is determined in both ovaries (42) and the difference between the two ovaries is proportional to the amount of LH injected. Significant depletion of ovarian ascorbic acid occurs after the injection of 0.125  $\mu$ g NIH-LH-S1. This assay has been used to measure FSH, pregnant mare serum gonadotropin (PMSG), and HCG as well as LH. Oxytocin, vasopressin, and ACTH have also been reported to cause depletion of ascorbic acid from the ovary so these substances must also be considered

possible sources of interference in this assay. Bogdanove and Gay (6) have modified the OAAD assay by treating the pseudopregnant rats with estrogen. This treatment prolongs the period (from 2.5 days to 2-3 weeks) during which the assay rats can be used and increases the sensitivity such that 0.01  $\mu\text{g}$  NIH-LH-S1 will cause a significant depletion in ovarian ascorbic acid.

Premature ovulation in hamsters has also been used to assay LH (62). The assay procedure is identical to that described above for FSH.

Recently, a bioassay has been developed which is capable of measuring levels of LH in serum. The redox bioassay is a histological technique which utilizes a change in the oxidation-reduction potential of a section of an ovary from a superovulated rat as the basis for quantification (52). The change in the oxidation-reduction potential of the tissue section is recorded by microdensitometry. This assay system does not appear to be influenced by hypophyseal hormones other than LH and provides a sensitivity of approximately 10 pg NIH-LH-S1. Levels of LH in serum as determined by the redox bioassay correlated very well with those determined by radioimmunoassay of the same samples. In addition, it appears that the redox state of ovarian tissue from superovulated rats is correlated with secretion of progesterone (29). This assay is quite expensive to perform compared to the radioimmunoassay but appears to quantify only biologically active LH.

Other bioassays capable of measuring levels of LH in serum have also been developed recently. For example, assays for LH have been developed which are based upon the ability of LH to stimulate production of progesterone in dispersed luteal cells (20) or to stimulate production of testosterone in dispersed testicular interstitial cells (9, 43). In addition to the added sensitivity, these bioassays are more precise and less expensive to conduct.

## F. HUMAN CHORIONIC GONADOTROPIN (HCG)

The biological activity of HCG and LH are similar. For this reason it may be assayed by any of the methods described above for LH. As with LH, one of the more sensitive bioassays for HCG is the hamster ovulation assay (62). The sensitivity for HCG in this assay is less than 0.1 IU.

A more classic bioassay for HCG is the vaginal cornification assay (22). The assay is conducted in immature rats and is based upon the ability of HCG to promote secretion of estrogens by ovarian follicles. The estrogens, in turn, cause cornification of the vaginal epithelium. The assay is conducted by giving 25-day-old female rats six subcutaneous injections (0.1 ml each) of HCG at 8-hour intervals. Vaginal smears are taken at 24,

36, and 48 hours after the last injection of HCG, stained with Giemsa stain, and observed under a microscope. The percentage of animals showing cornified smears after the treatment is compared to the response noted in rats receiving standard doses of HCG.

#### G. PREGNANT MARE SERUM GONADOTROPIN (PMSG)

The classic bioassay used to quantify PMSG is the immature rat ovarian weight assay first described by Cole and Erway (11). Immature rats (25 days of age) are given three intraperitoneal injections of the substance to be tested at 24-hour intervals. Twenty-four hours after the last injection the ovaries are removed, the bursa is dissected away, and the ovaries are weighed. The weight of the ovaries is proportional to the amount of PMSG injected. The sensitivity of this assay is approximately 2 IU of PMSG.

The hamster ovulation assay can also be used to quantify PMSG; its sensitivity is approximately 1 IU of PMSG.

#### H. PROLACTIN

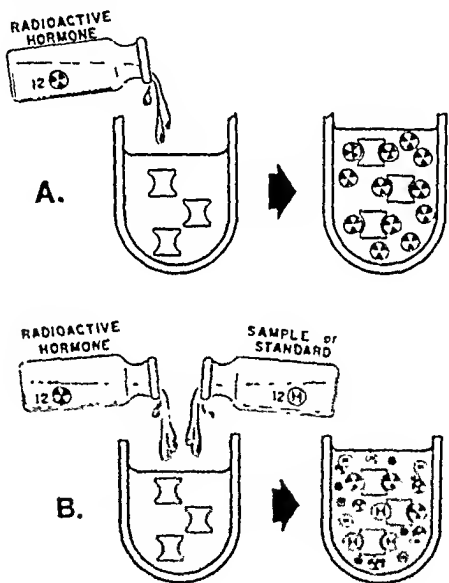
The classical bioassay for prolactin is the pigeon crop-sac assay (34). The assay is conducted by injecting standard prolactin or test material into pigeons daily for 4 days. The birds are killed on day 5, the crop-sac is dissected free, and weighed. Alternatively, the surface area of the crop-sac may be used as the basis for quantification. It appears that quantification based upon surface area may increase the precision of this assay. The original assay utilized the intramuscular route of injection and provided an assay sensitivity of approximately 2 IU of prolactin. However, it is possible to increase the sensitivity of the assay approximately 100-fold by injecting the standard prolactin or test material intradermally in an area over the crop-sac.

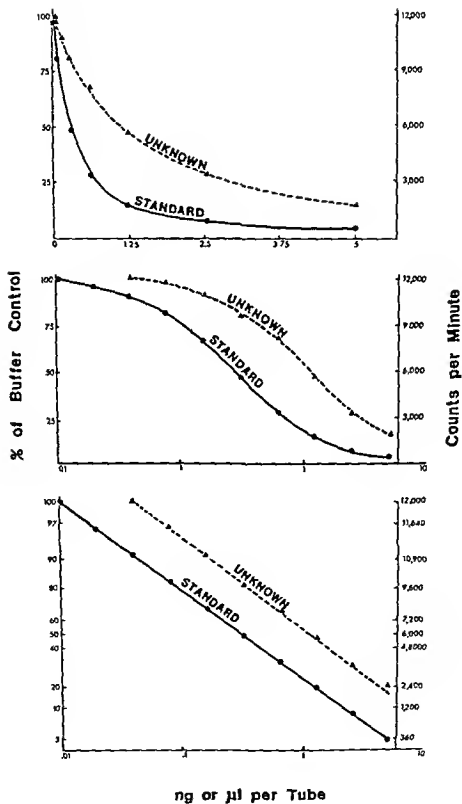
### III. Radioimmunoassay

Although it has been possible to quantify reproductive hormones under some conditions using biological assays, in general, these procedures were expensive, tedious, and too insensitive to detect the circulating levels of hormones. The methods used for the quantification of the reproductive hormones have undergone a virtual revolution since the initial observations of Berson and Yallow that antibodies against insulin could be used to develop a method for the quantification of this hormone. The observation that normal plasma or serum contained a protein capable of binding



radioactive adrenal steroids and progesterone (12, 55) was followed by the development of sensitive assay systems for these steroid hormones (46). The general principles of radioimmuno- and other competitive protein binding assays are the same. The basic concept of radioimmunoassay is described in Fig. 1. These assay systems are based on the ability of non-radioactive hormones to compete for binding sites on the protein and to prevent or displace the binding of radioactive hormone. When the quantity of binding protein and radioactive steroid is maintained constant, the inhibition of binding of radioactive hormone to the binding protein





a function of the quantity of nonradioactive hormone present in the sample or standard (Fig 2)

The successful development of a radioimmunoassay for insulin was followed by development of radioimmunoassays for a number of protein hormones including the gonadotropins and prolactin. It was found subsequently that steroid and peptide hormones could be used as haptens for production of specific antibodies. These observations led to the development of radioimmunoassays for all of the major steroid and peptide hormones. This procedure had such a major impact on quantitative endocrinology that it merits detailed consideration.

## A PRODUCTION OF ANTIBODIES

The antigenicity of any molecule is a function of its size, chemical composition, and the degree of foreignness to the animal being immunized. Large protein hormones (10,000–100,000 MW) are usually quite antigenic, while small peptide hormones (<2000 MW) and steroids are not antigenic under normal conditions. The number of species which have been immunized for production of antibodies to hormones is quite extensive and includes rabbits, guinea pigs, sheep, goats, rats, monkeys, horses, cattle, and turkeys. Rabbits and sheep have been used most frequently due to the low costs associated with their procurement and care and the ease of harvesting the antiserum. Large quantities of antiserum are unnecessary since a few milliliters of a high-titered antiserum are sufficient for millions of radioimmunoassay determinations.

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FIG 2 Three routine methods for depicting protein binding and/or radioimmunoassay data. The data depicted are the same in all three panels and represent inhibition curves obtained with varying quantities of a standard or sample (unknown) such as serum. In the upper panel the nanograms of standard or microliters of unknown ( $\times 10^3$ ) are plotted on an arithmetic scale on the abscissa. The counts per minute or percentage of radioactivity present in the buffer control tubes has been plotted on the ordinates. To calculate the percentage of the binding noted in buffer control tubes the quantity of radioactivity in tubes to which no standard or unknown has been added (buffer control tubes see first reaction Fig 1) is assigned the value of 100% and all other data are expressed as a percentage of this value. The data in the middle panel are plotted in the same manner on the ordinate. However the quantities of standard or unknown have been plotted on a log scale on the abscissa in both the middle and lower panels. In the lower panel the data on the abscissa have been subjected to a logit transformation. The method depicted in the upper panel is usually used for plotting protein binding data. When radioimmunoassay data were plotted the method depicted in the middle panel was used and the advent of computer analysis brought about the method depicted in the lower panel which allows easy analysis of parallelism between inhibition curves and assessment of linearity. It is not possible to evaluate either linearity or parallelism using the procedure depicted in the upper panel.

The quantity of antigen used for immunization depends upon a number of factors including: (1) the species to be immunized; (2) the purity of the antigen; (3) the characteristics of the antigen (i.e., whether it is a pure protein hormone or whether it is a steroid hormone which has been linked to a protein carrier); and (4) the method to be used for immunization. The methods used for immunization are as numerous as the different laboratories producing antibodies. However, it seems apparent that the method involving multiple interdermal injections (60) probably makes the most efficient use of valuable antigens and results in useful antibody production in a shorter time period.

Several considerations are important for the development of antibodies to small molecular weight hormones. It is necessary for these substances to be covalently linked to a large molecular weight protein in order for them to be antigenic. In the case of steroid hormones the site on the molecule through which it is attached to protein determines the specificity of the antibody (47). In order for a steroid-protein conjugate to be maximally antigenic there should be at least 20 steroid molecules attached to each molecule of protein.

## B. RADIOACTIVE ANTIGEN

The second component necessary for all radioimmunoassays is a suitable form of the antigen which can be easily quantified and distinguished from the naturally occurring hormone in the standards and test substances. Radioactive forms of the antigens are most commonly used. Radioactive iodine is incorporated routinely into the tyrosyl residues of protein molecules for this purpose. Radioiodination can be accomplished in a number of ways. However, the two most useful techniques are the chloramine-T procedure (24) and the lactoperoxidase procedure (59). The chloramine-T procedure is generally used for the radioiodination of protein hormones for radioimmunoassay, while the lactoperoxidase procedure is generally used for the radioiodination of proteins used in radioreceptor assays. The chloramine-T procedure is simple and produces compounds with high levels of radioactivity, which makes them very useful for radioimmunoassay (38). However, receptor-binding capability is often lost due to chemical damage of the hormone molecule. The lactoperoxidase procedure is milder and usually associated with a higher residual biological activity of the radioiodinated preparation. Small peptide hormones which contain a tyrosyl or histidyl residue can usually be radioiodinated using procedures similar to those used for large protein molecules. It is not possible to radioiodinate most steroid molecules directly. Most investigators have purchased tritiated forms of these molecules from

commercial sources. Others have covalently linked a tyrosyl or histidyl molecule to the steroid molecule so that direct radioiodination is possible (8, 47).

### C. METHODS FOR SEPARATION OF ANTIGEN-ANTIBODY COMPLEXES FROM THE ANTIGEN

The third important component of a radioimmunoassay system is an optimal procedure for separation of free antigen from antigen bound to antibody (Fig. 1). One of the major problems in the development of radioimmunoassays has been the selection of this procedure. This problem has been solved in a variety of ways, particularly in the case of the peptide and steroid hormones. The ideal method should provide a clean separation of these compounds, yet be unaffected by serum or other non-specific substances in the reaction mixture. In addition, the method should be rapid, simple, reproducible, and inexpensive. The methods of separation most commonly employed are (1) solid-phase antibodies, (2) solid-phase adsorption of antigen, (3) chemical precipitation of antigen-antibody complexes, and (4) immunoprecipitation of antigen-antibody complexes.

Early radioimmunoassays for LH (10) and estradiol (1) used polystyrene tubes which had the antibody coated on the interior surface. In this procedure the sample or standard and the radioactive hormone are allowed to react in the antibody-coated tube and the reaction is terminated by decanting the reaction mixture. For the assay of LH the residual radioactivity in the reaction tube (radioactive antigen bound to antibody) was quantified. For estradiol the reaction mixture was decanted into a liquid scintillation vial and the nonantibody bound tritiated estradiol was quantified. This procedure is very simple, but wastes antibody and lacks precision. In addition, when this procedure was used to quantify hormones in unextracted serum there appeared to be a decrease in the quantity of antibody bound to the tube due to nonspecific exchange with serum proteins. Mikhail *et al.* (41) used a suspension of polymerized antiserum which could be centrifuged directly to effect separation of free radioactive hormone from that bound to antibody. This assay procedure was also simple, but wasted antibody.

Adsorption of the unbound steroid or peptide hormone to insoluble particles such as charcoal, Florisil, Fuller's earth, tale, and similar compounds has also been used to separate free antigen from that bound to antibody (13). Several disadvantages are inherent in this method of separation. Most of the adsorbents have some affinity for antigen-antibody complexes as well as for free antigen resulting in removal of some of the

complexes from the reaction mixture. In addition, excessive amounts of adsorbent are generally used to effect a rapid separation. This necessitates critical timing of the reaction so that the contents of each tube receive the same exposure to adsorbent. Unfortunately, some adsorbents have a greater affinity for antigens (particularly steroid hormones) than do some antibodies. Since excessive amounts of adsorbents are used, considerable radioactive hormone may be "stripped" from the antibody during an extended reaction. Temperature is also critical since it influences the dissociation of antigen-antibody complexes. Therefore, most investigators have effected separation at 4°C to limit dissociation. It is very difficult to use adsorption procedures for the development of reliable radioimmunoassays.

Chemical means to precipitate antigen-antibody complexes have also been used to separate free radioactive steroid from that which is bound to antibody. Mayes *et al.* (37) have used 50% saturated ammonium sulfate to precipitate steroid-antibody complexes leaving the free steroid in solution. Polyethylene glycol has also been used very successfully to precipitate a variety of antigen-antibody complexes (16). Both of these procedures are performed very rapidly and easily but are not useful for large molecular weight proteins.

Under conditions for radioimmunoassay there is always an excess of hormone (antigen) in the assay tube and under these conditions an immunoprecipitate will not form. However, the precipitation of soluble hormone-antibody complexes by the addition of a second antibody (double antibody radioimmunoassay) to yield insoluble immune complexes has proved very successful and is the procedure used for most protein hormones. The second, precipitating, antibody is prepared against a gamma globulin fraction from the species in which the first, hormone-specific antibody was prepared (40). For example, if the antibody against the hormone is prepared in rabbits, then anti-rabbit gamma globulin might be produced in a sheep. In order to ensure the formation of visible precipitate, nonimmune serum (as a source of gamma globulin) from the species in which the first antibody was produced is added to the reaction mixture in a final dilution of 1:50 to 1:2000. This method of separation has certain disadvantages. It is relatively slow in comparison to other separation techniques and, when measuring hormones in unextracted serum, immunological components in the serum may interfere with the immunoprecipitation reaction if the conditions are not carefully controlled. Despite these disadvantages, the second antibody method of separation is preferred by many investigators owing to mild conditions required to effect a clean separation and high reproducibility of the method. In addition, the double antibody procedure is the only separation technique which is

reliable and efficient for all radioimmunoassay systems (i.e., it is universal). It has also been possible to insolubilize the second antibody by covalently linking it to Sepharose which allows the addition of large excesses of anti-gamma globulin and results in shortening the reaction time of the second antibody precipitation step to as little as 30 minutes (15). This later procedure has all of the advantages of both the double antibody and other separation methods.

#### D. VALIDATION OF RADIOIMMUNOASSAY SYSTEMS

The largest single problem in the development of radioimmunoassay systems is adequate demonstration that the assay measures only the substance which it is intended to measure. This characteristic is known as the specificity of the assay system. Specificity may be defined as freedom from interference by substances other than the one to be measured. Specificity of a radioimmunoassay system depends upon the unique combination of antibody and the radioactive antigen. Nonspecificity usually results from impurities in the antigen used for radioiodination or impurities in the immunogen used to produce the antibody. This is a problem particularly with the large molecular weight proteins. For example, pituitary hormones are purified from crude extracts of thousands of pituitary glands. Therefore, it is virtually impossible to eliminate all of the contaminating substances from any given preparation of a single hormone. This problem is not nearly so important in assays developed for steroid hormones or small molecular weight peptides since these compounds can be purified totally or produced synthetically.

The steps necessary to validate a radioimmunoassay are quite easy to understand. For example, if one is developing a radioimmunoassay for LH it is first necessary to demonstrate that other pituitary hormones do not interfere with the assay. Second, if one wishes to use that assay for measurement of LH in serum, it is necessary to demonstrate that components in serum, other than LH, do not influence the assay system. It is possible to demonstrate that other pituitary hormones do not influence the radioimmunoassay for LH by adding purified preparations of these hormones to the assay system and ascertaining their ability to inhibit the binding of the radioactive LH to the antibody. If the preparations of these hormones do inhibit the binding of LH to its antibody then the inhibition curves should be parallel to those obtained with the LH standard (see Fig. 3). Parallelism between the inhibition curve obtained with an unknown sample and that obtained with a preparation of LH suggests that it is the same substance within each of these materials which is binding to the antibody. If it were a different substance the kinetics of binding

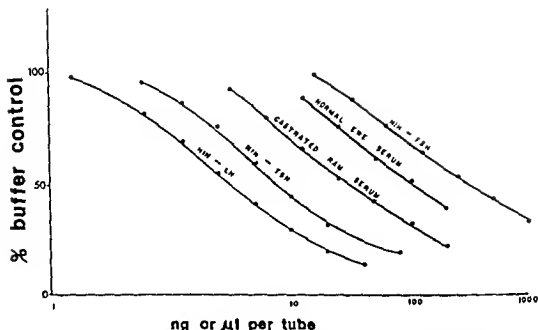


Fig. 3. Inhibition of binding of radioactive LH to antibody with varying quantities of pituitary preparations containing high levels of LH, TSH, and FSH or serum from a castrated ram or a normal ewe. The inhibition curves are parallel to each other.

would likely not be the same as that noted for LH and it would produce a nonparallel inhibition curve. Although a lack of parallelism between inhibition curves obtained with test substances and those obtained with preparations of LH would suggest an invalid, nonspecific assay, parallelism is not adequate proof of specificity. For example, LH, FSH, and TSH are each composed of two subunits designated as  $\alpha$  and  $\beta$ . The  $\alpha$  subunit is identical for all three hormones while the  $\beta$  subunit is unique for each hormone. Since a single antigenic site is usually relatively small (4–10 amino acids), it is possible for antibodies to be produced against antigenic sites contained within the  $\alpha$  subunit. Although such an antibody would have immunological specificity for that particular antigenic site and inhibition curves obtained with preparations of LH, FSH, and TSH would all be parallel, this particular assay system would not be hormone-specific. Therefore, it would not be valid for the measurement of LH. If partially purified preparations of FSH and TSH produced inhibition curves which were parallel to the one obtained with LH it would be necessary to determine if the inhibition was caused by LH contamination of these preparations. This can be ascertained by comparing estimates of the LH content of each of the preparations as determined by bioassay with that determined by radioimmunoassay (Table I). If there is good agreement between these estimates, then this is usually sufficient proof that the material that is inhibiting the assay is, in fact, LH and that the assay is specific



TABLE I

Luteinizing Hormone Content of Ovine Pituitary Preparations

Preparation	Biological activity (U/mg)				Bioassay LH <sup>d</sup>
	TSH <sup>a</sup>	FSH <sup>b</sup>	LH <sup>c</sup>	LH RIA	RIA LH
NIH-LH	0.054	0.021	0.82	0.86	0.97
NIH-FSH	0.008	1.37	0.017	0.016	1.06
NIH-TSH	1.25	—	0.37	0.43	0.86
NIH-Prolactin	0.0005	0.008	0.0003	0.0003	1.00
NIH-Growth hormone	0.012	0.11	0.034	0.027	1.26

<sup>a</sup> TSH determined by the thyroid <sup>125</sup>I uptake in chicks.<sup>b</sup> FSH determined by the HCG augmentation ovarian weight assay.<sup>c</sup> LH determined by the ovarian ascorbic acid depletion assay.<sup>d</sup> These data suggest excellent agreement between the bioassay and radioimmunoassay estimates of LH concentration even in those preparations with high FSH, TSH, prolactin, and growth hormone contents.

(see Table I). However, as discussed previously, many bioassays are not reliable for estimating low levels of contamination in highly purified pituitary preparations due to a lack of specificity. In addition, some purification procedures may destroy the biological activity of a hormone molecule but leave its immunological activity intact. Therefore, if there is a lack of agreement between radioimmunoassay and bioassay estimates of hormone potency the next step is to characterize the interfering substance(s). This is usually accomplished by electrophoretic and gel filtration procedures (32). These procedures have shown that the bioassay estimates were erroneous in most cases. If large quantities of a purified preparation of a pituitary hormone do not appear to influence the assay system then it is usually safe to conclude that the assay is not influenced by this hormone (see NIH-growth hormone and NIH-prolactin, Table I).

If the assay is to be used to measure LH in serum (or plasma) then inhibition curves obtained with differing quantities of serum should also be parallel to those obtained with the LH standards. In addition, it should be possible to add varying amounts of LH to a constant quantity of serum and to quantitatively assay each of these additions (see Fig. 4). This suggests that other components of serum do not interfere with the estimation of LH. It should also be possible to remove the source of the hormone, i.e., in the case of LH one would remove the pituitary gland, and show that blood levels of the hormone in question become nondetectable. It should also be possible to reproduce various biological data which have been reported previously. In the case of LH, for example, it should

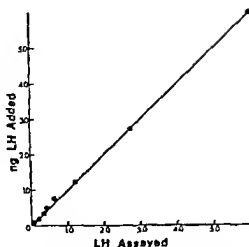


FIG. 4. A comparison of the quantities of LH added to 200  $\mu$ l of ovine serum and the values obtained following quantification by radioimmunoassay. The level of endogenous LH in the serum has been subtracted from all estimates obtained by radioimmunoassay. If perfect agreement had occurred between the quantities of LH added and those measured, the regression analysis would have indicated a correlation coefficient of 1.00, a slope of 1.00, and an intercept of 0.

be possible to show that castration results in an elevation of LH in serum while steroid therapy should reduce these levels. Procedures similar to those described for LH must be followed to validate radioimmunoassays for other protein hormones.

After the specificity of an assay has been demonstrated it is then necessary to describe some of the important parameters of the assays' performance. One of the most important parameters is its sensitivity. Sensitivity is defined as the least quantity of hormone that can be distinguished reliably from no hormone. Another criterion which should be evaluated is reproducibility which is defined as the extent to which an estimate is duplicated upon repeated measurements. The final criterion that is important to describe is accuracy, which is defined as the extent to which the mean of a number of measurements of a substance agrees with the exact amount of the substance which is present. Accuracy is often determined by comparing radioimmunoassay estimates with actual values determined by some other procedure (i.e., gravimetrically, see Fig. 4).

When developing a radioimmunoassay for steroid hormones the general principles are similar to those used for evaluating assays for protein hormones. It is necessary to demonstrate that steroid hormones other than the one to be measured do not interfere with the radioimmunoassay system. The potential for interference by steroids with closely related structures can be evaluated by determining their ability to inhibit binding of the radioactive steroid to the antibody. With this information it is usually apparent which steroids are likely to cause problems in the assay. For

TABLE II

Comparison of Serum Concentrations of Progesterone by Radioimmunoassay with and without Purification of the Sample by Thin-Layer Chromatography

Species	Number of samples	Range in concentration (ng/ml)	Correlation <sup>a</sup> coefficient	Slope
Ovine	37	0.20-2.00	0.97	1.03
	45	2.00-16.00	0.94	0.97
Bovine	12	0.10-11.0	0.98	0.93
Equine	12	0.10-20.4	0.98	0.86
Canine	12	0.10-15.6	0.86	1.19
Human	12	0.10-15.1	0.98	0.87

<sup>a</sup> If there were perfect agreement between the values obtained before and after purification of the progesterone in the sample the correlation coefficient would be 1.00 and the slope of the regression line would be 1.00. The data depicted indicate that there was excellent agreement between the values obtained with and without purification.

example, dihydrotestosterone might react strongly with an antibody to testosterone while estradiol, progesterone and cortisol have no significant ability to inhibit the binding of testosterone to its antibody. The next step in validation of a steroid radioimmunoassay should be to compare values obtained on samples before and after the hormone to be measured has been purified using a chemical procedure (see Table II). The methods used for chemical purification are usually column, thin-layer, or paper chromatography. A final proof of the specificity of the assay is to compare values obtained by radioimmunoassay on a number of samples with values obtained on those samples using other techniques (Table III). Once you have proved the specificity of a steroid radioimmunoassay system it is also necessary to describe the assay system in terms of its sensitivity, reproducibility, and accuracy.

#### IV. Protein-Binding Assays

The basic principles of the protein-binding assays are similar to those of the radioimmunoassay except that a protein with a high affinity for the hormone is used rather than an antibody. Protein-binding assays for steroid hormones have largely been replaced by radioimmunoassay due to a greater specificity and higher affinity of the antibodies for the steroid. Only recently has the protein-binding technique been extended to the quantification of protein and glycoprotein hormones.

TABLE III

Levels of Progesterone in Serum (ng/ml)  
Determined by Three Methods\*

Sample	Radio-immunoassay	Competitive protein binding	Double isotope derivative
147	3.7	3.1	3.6
165	4.9	5.3	5.0
146	7.4	3.1	7.7
164	4.2	4.2	7.0
166	9.1	11.6	8.1
75	8.6	8.2	9.6
144	11.2	10.5	14.7
151	19.4	24.2	20.7
167	31.8	20.7	30.7

\*These data indicate excellent agreement regarding the progesterone concentrations determined using the three different procedures.

## A. STEROID HORMONES

The advent of protein-binding assays for quantification of steroid hormones (46) was a major advance. This was the first technique that did not require a large sample volume and extensive purification of the steroid prior to final quantification. Although these techniques were first reported for quantification of adrenal corticosteroids (46) similar assays were rapidly developed for progestins (45), androgens (61), and estrogens (28). The binding proteins used for sex steroids were obtained from serum or target organs. Receptor molecules in target tissues are generally more specific and may have slightly higher affinities than binding proteins in serum.

Corticosteroid-binding globulin (transcortin) has been used for the assay of progestins (45) as well as corticosteroids (46). Transcortin is an  $\alpha_1$ -globulin present in the serum of most mammals and has a relatively high affinity for progesterone. Levels of transcortin in serum increase during pregnancy due to estrogenic stimulation. Therefore, estrogen therapy has been used to increase the level of transcortin in donor animals, effectively increasing the number of assays that may be performed with a given volume of serum. Donor animals have also been given large doses of synthetic glucocorticoids, such as dexamethasone or flucortisone, prior to the collection of blood to depress endogenous glucocorticoids which decreases the saturation of transcortin with endogenous steroids. Other investigators collect blood from untreated donors and remove endo-

genous steroids by adsorption with charcoal, Fuller's earth, or Florisil. This treatment removes steroid molecules from previously saturated binding sites.

In order for steroid molecules to bind to transeortin they must have ketone groups at position 3 and 20 and a double bond between carbons 4 and 5. Hydroxyl groups at positions  $11\beta$ ,  $17\alpha$ , and 21 of the pregnane nucleus result in increased affinity of transeortin for the steroid molecule. This indicates that transeortin is not very effective for measuring levels of natural occurring progestins, other than progesterone and  $17\alpha$ -hydroxyprogesterone.

In addition to transeortin, a progesterone-binding protein has been reported in plasma from pregnant guinea pigs (7). This protein appears to be much more specific, has a higher binding affinity for progesterone, and is present in higher concentrations in serum than transeortin. These three properties make the progesterone-binding protein more suitable for the assay of progesterone than transeortin.

Sex hormone-binding globulin (SHBG) was first used for the assay of testosterone by Horton, Kato, and Sherins (23) and Mayes and Nugent (36). SHBG is present in human plasma and appears to be present in higher concentrations in women than in men. Levels of SHBG are elevated during late pregnancy. It appears to bind mainly androgenic compounds with a  $17\beta$ -hydroxyl group, but also binds estradiol- $17\beta$  (18, 37). Therefore, if quantification of a single steroid in serum is desired, rather than quantification of total  $17\beta$ -hydroxysteroids, the substance of interest must be purified (separated) by chromatography prior to assay.

Although SHBG has been used to measure estradiol- $17\beta$  it appears that a specific estrogen-binding protein found in plasma of pregnant rats may be more suitable. This protein has a high affinity for estrone and estradiol- $17\beta$  and does not bind testosterone (56). However, the most commonly used binding protein for the assay of estrogens is obtained from the cytosolic fraction of a uterine homogenate (25). This estrogen-binding protein is stable indefinitely when stored in liquid nitrogen and has a high affinity and specificity for estradiol- $17\beta$  and estrone (28).

## B. GONADOTROPIC HORMONES

Although several competitive binding assays for protein hormones have been reported, it has not been possible to universally adapt these for measurement of hormones in serum. The lack of sensitive, competitive-protein binding (receptor) assay systems for gonadotropic hormones appears related to the affinity with which receptors bind gonadotropins ( $K_a \sim 1 \times 10^{10}$  moles/liter) (9). This affinity is relatively low when com-

pared to the affinity with which antibodies are capable of binding gonadotropic hormones ( $K_a \sim 1 \times 10^{12}$  moles/liter) (19). The lower affinity results in inadequate sensitivity of receptor assays for measurement of gonadotropins in serum. If it is assumed that gonadotropin molecules which bind to receptor are biologically active then this assay has the advantage of quantifying only *biologically active* hormones. Conversely, quantification of gonadotropic hormones via radioimmunoassay does not reflect only biologically active hormones, but is a measure of immunologically active fragments or subunits of the gonadotropin as well. Therefore, it is important that samples to be quantified by radioimmunoassay be collected and processed in as mild a manner as possible.

Radioreceptor assays have been developed for prolactin (54), LH (31), FSH (53), and HCG (9, 30). These assays employ membrane fractions containing the gonadotropin receptors as binding agents and use radioiodinated gonadotropins for quantification. Preservation of receptor binding activity of the hormone after radioiodination has been a major problem hindering the development of radioreceptor assays. Modification of the original chloramine-T procedure (24) to make the reaction conditions much milder (31, 53) or use of the milder lactoperoxidase procedure (59) has resulted in production of radioiodinated gonadotropins which bind to receptor. The sensitivity of radioreceptor assays far exceeds the sensitivity of any of the classic bioassays and approaches that of radioimmunoassay and, in some cases, these assays have been used to measure serum levels of gonadotropins when they are present in large quantities, i.e., HCG and human placental lactogen in pregnant women.

## V. Summary

Development of our understanding of the complex hormonal interrelationships which regulate reproduction in domestic animals has been linked directly to our ability to quantify the hormones involved. Initially, both the sex steroids and the gonadotropic hormones were quantified with bioassays. These procedures were tedious, expensive, lacked specificity, and were not sufficiently sensitive to measure the small quantities of these hormones in peripheral blood. However, the data obtained with these procedures have generally proved to be correct. Direct chemical assays of the sex steroid hormones soon replaced the bioassay procedures. These assays were more sensitive and very specific but were still tedious and expensive to perform.

During the last decade our knowledge of the endocrinology of reproduction has increased dramatically due primarily to the development of

competitive protein-binding and radioimmunoassay procedures. The procedures have proved to be simple to perform, inexpensive, sensitive, and specific. Therefore, it has been possible for the first time, to quantify reliably all of the major reproductive hormones in small quantities of plasma or serum. This capability has resulted in the complete characterization of the hormonal changes which occur during different reproductive states and forms the basis for the chapters which follow.

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# 6

## Mechanism of Action of Sex Steroid Hormones in the Female

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### I. Introduction

Steroid sex hormones are transported via the blood stream to all parts of the body, yet these compounds stimulate only certain tissues and organs. To early workers in the field of drug and hormone action, this simple fact

suggested that those organs which did respond to a given hormone must contain recognition units or receptors. Otherwise, hormones would have equal effects in all cells. In addition to this *a priori* reasoning for the existence of receptors, it was also known that hormones acted at very low concentrations, e.g.,  $10^{-10}$ – $10^{-8}$  M. Thus, it was assumed that in order to detect these extremely low quantities of steroids a receptor should have a high binding affinity for these compounds. Early investigations of steroid hormone receptors were hampered since radioactive steroids of high specific activity were not available. Thus, specific binding of  $^3\text{H}$ -steroid to a receptor could not be measured because the number of nonspecific binding sites for the  $^3\text{H}$ -steroid was very great. Jensen and his co-workers (51) made a major contribution to the study of the mechanism of hormone action when they synthesized  $^3\text{H}$ -estradiol which had a very high specific radioactivity. It was then possible to observe differential accumulation and retention of  $^3\text{H}$ -estradiol by the uterus, vagina, pituitary, and other target organs (51, 58, 76). Since that time, similar steroid–target organ interactions have been observed for all steroid hormones. In this chapter we will be concerned with these receptor–steroid interactions and how they stimulate and control cellular activity. A general scheme for these interactions is shown in Fig. 1 and is to be used as a guide to the

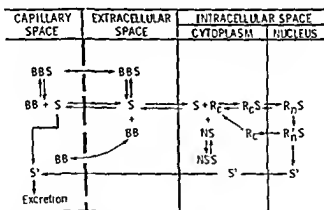


FIG. 1 General scheme for steroid receptor binding. Steroid hormones in the blood may be free (S) or bound to blood binders (BBS). Entry into the extracellular space by either of these forms can result in the diffusion of S into the cytoplasm. The steroid binds to specific cytoplasmic receptor sites ( $R_c$ ) or to non-specific sites (NS) to form bound cytoplasmic complexes ( $R_cS$ ) and (NSS). The  $R_cS$  complex undergoes translocation to the nucleus ( $R_nS$ ) where it probably undergoes processing or metabolism to inactive forms ( $R_nS$ ; please note: the formation of  $R_nS$  complex is only one of the possible pathways by which  $R_cS$  could be inactivated).  $R_c$  is replenished via a recycling and/or resynthesis mechanism and the altered steroid ( $S'$ ) diffuses out of the cell and is excreted (see text for details of these reactions).

concepts and nomenclature which will be presented in more detail throughout the chapter.

## II. Blood Binding, Metabolism, and Tissue Interactions

The interaction of sex steroids with intracellular receptors depends on the quantity of free hormone available for entry into target cells. This parameter fluctuates as a function of several variables which are represented in Fig. 1 and are the subject of the following section.

### A. BLOOD BINDING OF STEROIDS

Sex steroids bind to many of the macromolecules found in blood (BB, Fig. 1). The affinity of these blood binders for steroids varies from very weak ( $K_d \sim 10^{-3} M$ ) to very strong ( $K_d \sim 10^{-10}$ – $10^{-6} M$ ) and they are frequently present in high concentrations (75, 106). Therefore, these proteins can restrict the amount of free hormone available for receptor binding and hence can be important in the control of steroid hormone action. Simple measurements of total steroid hormone concentration in the blood may lead to erroneous conclusions regarding the level of hormone stimulation in the tissues. One such binding molecule is testosterone-estrogen binding globulin also called  $\alpha$ -fetoprotein ( $\alpha$ -FP) which binds both testosterone and estradiol with equal affinity,  $K_d \sim 10^{-10} M$  (75, 106). During neonatal and prepubertal life of the rat,  $\alpha$ -FP gradually declines from very high values in the newborn to very low values just before and after puberty (91). Therefore, the quantity of free sex steroid would gradually increase as the concentration of  $\alpha$ -FP declines. In this manner, increasing concentrations of steroid are available for cellular interactions.

Binders of sex steroids which do not demonstrate specific binding may also be significant in the physiology of steroid hormones. Serum albumin, a component of vascular and extracellular spaces, has a relatively weak affinity for estrogen ( $K_d \sim 10^{-4}$ – $10^{-5} M$ ), but is a significant estrogen binder (115). The concentration of serum albumin in the blood is  $\sim 4\%$  ( $\sim 7 \times 10^{-4} M$ ). At this concentration it can alter the equilibrium distribution of steroids in cells and, hence, is important in considering the relative potencies of hormones. By way of example, the  $K_d$  values for complexes of  $E_2$  and  $E_1$  with the estrogen receptor at  $4^\circ C$  are  $10^{-10}$  and  $10^{-9} M$ , respectively. Therefore,  $E_2$  should be ten times more potent than  $E_1$ . However, it has been demonstrated that these two hormones are equally effective in the stimulation of early uterotrophic events, i.e., events

such as water imbibition, RNA, and protein synthesis, which occur during 1–6 hours after injection (3, 7, 46). This discrepancy between the predicted potency and the observed potency results from the differential binding of these steroids to plasma components (6). The affinity of blood binders, such as serum albumin, for  $E_2$  is ten to a hundredfold greater than that for  $E_1$  (6). Therefore, when these steroids are injected, the amount of  $E_1$  which is free and available for tissue interaction is much greater than the amount of free  $E_2$ . These elevated levels of  $E_1$  result in approximately equal receptor binding and equal stimulation of early uterotrophic events (3, 7, 46). (See Chapter 4 for symbols of steroids.)

As shown in Fig. 1, blood binder steroid complexes, BBS, may enter the extracellular compartment. If this occurs it is possible that these complexes serve to direct hormonal signals toward organs with protein permeable vascular beds (57). It has been shown that estradiol increases the accumulation and retention of albumin by the uterus while not affecting such nontarget tissues as psoas muscle, liver, or brain (84). This accumulation and retention of albumin, and thus presumably of albumin-bound estrogen may be involved in establishing levels of *total* estrogen in the uterus that are greater than those of the systemic circulation and may be maintained at higher levels for longer periods of time (83).

## B. METABOLISM AND STEROID TISSUE INTERACTIONS

The quantity of steroid available for receptor binding *in vivo* depends not only on blood binding relationships but also in the rate of metabolism and excretion of that hormone (Fig. 1,  $S \rightarrow S'$ ). Thus, the metabolic clearance rate, *MCR*, or that volume of blood required per unit time to remove the administered hormone from the body, is very important to considerations of biological activity. A rapid *MCR* will reduce the exposure time of a hormone to its receptor, whereas a slow *MCR* will increase exposure time. These factors must be considered in predicting or analyzing the biological effectiveness of any hormone.

Estrogen and progesterone do not depend on the metabolism conversion for biological activity (51, 100). That is, once receptor steroid complexes have formed after steroid entry into the cell, these complexes are functionally active. However, caution should be exercised in assuming that metabolism is unimportant in all cases since it has been shown that the conversion of testosterone to 5 $\alpha$ -dihydrotestosterone is a requirement for androgen action in some male sex accessory structures (1, 17, 59).

In addition, metabolism may be important in the elimination of steroids from target tissues. Gurpide and Welch (43) have demonstrated that estradiol and estrone undergo extensive interconversion in human endo-

metrium and that estradiol is metabolized to estrone before it is released from the tissue (also see 42, 58).

### III. Cellular Accumulation and Cytoplasmic Binding of Steroid Hormones

#### A. STEROID UPTAKE BY CELLS

The entry of steroids into cells from the extracellular space appears to occur by diffusion (Fig. 1). Until recently, studies of steroid uptake have involved *in vivo* or *in vitro* exposure of target and nontarget tissues to labeled steroid for extended periods of time (41, 51). Such studies, which invariably suggest that a saturable component is involved in steroid uptake, more accurately reflect the retention of steroid after its interaction with receptors and do not measure the rate of entry of steroid into cells. Gurpide and Welch (43), using a double isotope steady-state perfusion technique, have shown that a number of steroids enter target and nontarget tissues with equal facility at a rate directly proportional to steroid concentration over a range of 0.2 to 5000 ng/ml (42, 43). Although these results do not eliminate the possibility of a carrier-mediated process, they are most easily interpreted in terms of simple diffusion. In an attempt to differentiate entry and retention, Peck *et al.* (83) have examined the *in vitro* uptake of  $^3\text{H}$ -estradiol into uteri under initial velocity and equilibrium conditions. These studies demonstrated that (1)  $R_c$  is not involved in the entry of estradiol into uterine tissue; (b) entry of estradiol is not dependent on metabolic energy; and (c) entry of  $^3\text{H}$ -estradiol is not inhibited by estrogenic agents such as DES; these studies support previous suggestions that estradiol initially partitions between medium and tissue (target or nontarget) in a nonspecific, passive manner.

#### B. RECEPTOR-STEROID BINDING IN THE CYTOPLASM

Subsequent to entry into target cells, steroids may either bind to the cytoplasmic receptor,  $R_c$ , to form a receptor steroid complex,  $R_cS$ , or associate with various low affinity binding sites, NS, within the cytoplasmic compartments (Fig. 1). Thus, retention of intracellular steroid under equilibrium conditions involves two types of binding sites: a limited number having a high affinity and marked specificity and a second class with low affinity but a very large capacity (32, 41, 52, 81, 83, 94). These two types of binding must be carefully differentiated if accurate measurements are to be made of the number of steroid-receptor complexes. Therefore,

the criteria listed below have been established for steroid hormone receptors (28).

## 1. Receptor Criteria

a. **FINITE BINDING CAPACITY.** As mentioned in the introduction, the biological response to a steroid hormone is a saturable phenomenon. If the formation of receptor-steroid complexes is obligatory for the production of a biological response, then the quantity of steroid receptor should be limited; hence a finite number of binding sites. This criterion should be met by the demonstration that the binding activity of interest can be saturated by its specific ligand.

b. **HIGH AFFINITY.** Steroid receptors are expected to have a high affinity for their respective hormones. This is anticipated because the blood levels of steroid are usually  $10^{-10}$ – $10^{-8}$  M. If a tissue is to respond to a hormone via a receptor mechanism in which the hormone associates with a receptor, then the receptor must have an affinity for the hormone which is in the range of physiological levels; otherwise no response would occur. These considerations, borne out for a host of target tissue receptors, do not rule out receptor interactions of weaker affinity if blood levels of steroids are high.

c. **STERIOD SPECIFICITY.** Generally speaking, a receptor is expected to display high affinity for a specific hormone or class of hormones. This specificity enables a given target cell to respond to a given hormonal signal without interference from other signals. Thus, hormones of the same physiological class should compete effectively for their receptor while not affecting other receptor systems and vice versa.

d. **TISSUE SPECIFICITY.** Only certain tissues and organs appear to be stimulated by the sex steroids. Classically these have been referred to as target organs, e.g., uterus, vagina, and mammary gland. If the effects of sex steroids on these target organs result from receptor-steroid interactions, then the quantity of receptor should be higher in target than in nontarget tissues.

e. **CORRELATION WITH BIOLOGICAL RESPONSE.** Implicit in all studies of macromolecules which bind steroids and meet the above criteria is the assumption that this binding results in a biological response. This criterion, the demonstration of receptor-dependent hormonal response, is not

often met and is the most difficult to establish. The quantity of nuclear bound estrogen receptor has been shown to be proportional to the stimulation of uterine growth responses (4, 7, 27, 28), and in some receptor systems it has been possible to show that in the absence of the cytoplasmic receptor no response to the hormone occurs (5, 18, 25, 35, 73, 104).

## 2 Physical Characteristics of Steroid Receptors

Estrogen and progesterone receptors are large macromolecules found in the soluble fractions of target tissues. Talwar *et al.* (110) first demonstrated that cytoplasmic fractions of rat uteri contained a macromolecule which binds  $^3\text{H}$ -estradiol. Subsequently, Toft and Gorski (113) showed that this receptor- $^3\text{H}$ -estradiol complex moves as an 8 S peak on sucrose density gradients. These pioneering studies led to extensive investigations of the physical characteristics of the steroid receptors which are summarized in Tables I and II. In general, steroid hormone receptors are large ellipsoid proteins which are found principally in the cytoplasm in the absence of hormone.

TABLE I  
Physical Properties of the Estrogen Receptor

Parameter <sup>a</sup>	Cytoplasmic		Nuclear
	Low salt	High salt	
Sedimentation coefficient (S)	7-9 <sup>b</sup>	4 <sup>c</sup>	4.5 <sup>1</sup>
Stokes radius (Å)	67	33	33
$f/f_0$	1.65	1.25	1.25
Axial ratio (prolate ellipsoid)	8.3	3.4	3.4
Isoelectric point	6.2	6.7	6.7
Molecular weight	200 000	60 000-100 000	60 000-100 000
Dissociation constant ( $K_d$ )	$10^{-10}$ - $10^{-8}$ M		$10^{-10}$ - $10^{-8}$ M

<sup>a</sup> All values used in this table are approximations based on the work of Gorski *et al.* (41), Giannopoulos and Gorski (36), Puga *et al.* (86), Erdos *et al.* (37), Chamness and McGuire (19), Jensen and DeSombre (53), Puga *et al.* (87), Stancel *et al.* (108), Notides and Nielsen (77).

<sup>b</sup> Sedimentation values vary depending on the ionic strength of buffer [Stancel *et al.* (108) and Chamness and McGuire (19)] but generally in low salt buffers the estrogen receptor will be approximately 8 S.

<sup>c</sup> Under denaturing conditions all forms of the receptor have an S value of 3.6 [Stancel *et al.* (108)].

<sup>1</sup> 4 and 5 S forms are also found in the cytoplasm under certain conditions [Notides and Nielsen (77), Jensen and DeSombre (53)].

TABLE II

Physical Properties of the Progesterone Receptor\*

Parameter	Monomeric forms		Dimer A-B
	A	B	
Sedimentation coefficient (S)	4.2	4.2	6
Stokes radius (Å)	55	63	
$f/f_0$	1.74	1.9	
Axial ratio	14	18	
Isoelectric point	4.5	4.0	
Molecular weight	110,000	117,000	227,000
Dissociation constant	$10^{-14} M$	$10^{-14} M$	

\* Taken from Schrader and O'Malley (99); Schrader *et al.* (101).

### 3. Receptor States in the Cytoplasm

The cytoplasmic compartment contains two forms of receptor: unfilled sites ( $R_e$ ) and filled sites ( $R_eS$ , Fig. 1). Until recently the evaluation of cytoplasmic receptor sites was hampered because assay methods only permitted the detection of  $R_e$  (unfilled sites). The measurement of total receptor was not possible and hence receptor concentrations were always underestimates. The demonstration that total receptor can be measured by  $^3H$ -estradiol exchange has permitted the evaluation of both  $R_eE$  and  $R_eS$  (nuclear receptor) under physiological conditions and *in vivo* (2, 23, 25, 26, 55). The exchange method for the determination of total receptor and the differentiation of filled and unfilled receptor sites is based on the temperature dependence of the rate of steroid dissociation. At 0° to 4°C,  $R_eE$  (filled sites) dissociate very slowly with a  $T_{1/2}$  of 20 to 30 hours (32, 98), whereas  $R_e$  (unfilled sites) readily saturate with  $^3H$ -steroid. However, at elevated temperatures, in addition to the saturation of  $R_e$  sites, previously filled  $R_eS$  readily dissociate and bind added  $^3H$ -steroid (2, 98).

The number of cytoplasmic filled sites,  $R_eS$ , is normally quite low since these associate rapidly with the nuclear compartment (116). This is often ignored by investigators and leads to a misinterpretation of the number of filled sites *in vivo*. Williams and Gorski (116) were first to measure the actual amount of  $R_eS$  in the uterus. They exposed uteri to  $^3H$ -estradiol *in vitro* and then homogenized the tissue in buffer which contained excess  $E_2$ . The excess nonlabeled  $E_2$  competes with the free and nonspecifically bound  $^3H$ -estradiol that is available at the time of homogenization from intra- and extracellular spaces for binding to unfilled  $R_e$  sites. Therefore, the quantity of  $^3H$ -estradiol bound to the receptor at the time of homogen-



ization can be assessed. By combining the use of excess steroid in the homogenization medium with the  $^3\text{H}$ -steroid exchange procedure, one can assess filled and unfilled sites in tissues exposed to unlabeled steroid *in vivo*. Therefore, when tissues are homogenized in the cold with excess  $^3\text{H}$ -steroid, unfilled sites will be filled with  $^3\text{H}$ -steroid while filled sites remain occupied with unlabeled steroid. By maintaining ice-cold conditions, unfilled sites can be determined. By briefly elevating the temperature after homogenization, those sites (filled and unfilled) will be measured. The difference between total sites, as measured by exchange, and unfilled sites, as measured in the cold, will represent those cytoplasmic sites filled by steroid *in vivo*.

A number of investigators have suggested cooperative behavior in the interaction of estradiol with the  $R_c$  (31, 32, 86, 98). However, under similar experimental conditions other laboratories have been unable to demonstrate cooperative binding (22, 36, 117). In a definitive work, Williams and Gorski (117) have studied the equilibrium binding of estradiol by uterine cell suspensions and whole uteri *in vitro*. They demonstrated simple binding behavior between 5 and 95% saturation of  $R_c$ . Together with the studies of Peck *et al.* (83), in which  $R_nE$  was shown to be a simple function of estradiol concentration, these studies demonstrate the simple equilibrium of estradiol with independent binding sites,  $R_c$ , in a noncooperative manner.

#### IV. Nuclear Binding of the Receptor-Steroid Complex

##### A. CYTOPLASMIC TO NUCLEAR TRANSLOCATION

The  $R_cS$  complex appears to move from the cytoplasm to the nucleus,  $R_nS$ , of target cells (Fig. 1,  $R_cS \rightleftharpoons R_nS$ ). This conclusion was derived from the work of Jensen *et al.* (52) and Shyamala and Gorski (103) who showed that with nuclear accumulation of bound estrogen there was a concomitant depletion of cytoplasmic receptor. The depletion of  $R_c$  and concomitant accumulation of  $R_n$  show temporal and stoichiometric relationships which fit with the concept of cytoplasmic to nuclear translocation (Fig. 2). The experiment shown in Fig. 2 was performed with the  $^3\text{H}$ -estradiol exchange assay which permits the measurement of the total quantity of  $R_c$  and  $R_n$ , and therefore reflects the true compartmentalization of  $R$  (2, 28, 56).

The concept of cytoplasmic to nuclear translocation is further substantiated by the observation that  $R_cE$  and  $R_nE$  complexes share similar dissociation constants, hormone specificities (85), and sedimentation co-

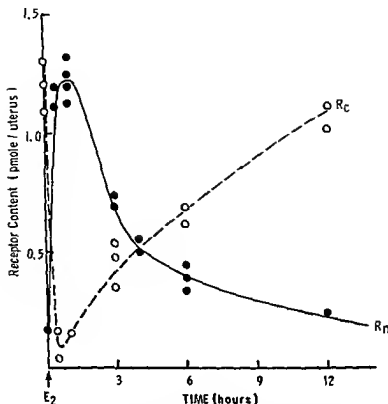


FIG. 2. The effect of estradiol on the quantities of estrogen receptor in the cytoplasm and nucleus. Immature rats were injected with 2.5  $\mu$ g of estradiol and the quantity of receptor in the cytoplasmic ( $\circ$ ) and nuclear ( $\bullet$ ) fraction were determined by the  $^3$ H-estradiol exchange assay

efficients under denaturing conditions (108). The translocation process also occurs as a function of fluctuating blood levels of estrogen during the estrous cycle (23). These observations, in addition to the thermodynamic considerations made by Williams and Gorski (117, see Section III,B,3), indicate that the  $R_nS$  complex is derived from the  $R_cS$  complex by a translocation process.

#### B. NUCLEAR ACCUMULATION AND RETENTION OF THE RECEPTOR-STEROID COMPLEX

The binding of the  $R_nS$  complex to nuclear sites probably involves at least two kinds of sites on chromatin (Fig. 3): nonacceptor sites, N, (nonspecific binding sites), which are in large numbers, serve to maximize the probability that nuclear binding of the  $R_nS$  complex will occur, and acceptor sites, A, which are limiting and in small numbers. The binding and retention of the  $R_nS$  complex to a small number of nuclear sites, possibly acceptor sites, is correlated with growth responses in the uterus

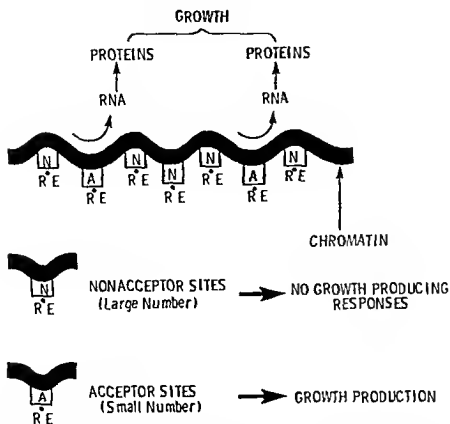


FIG 3. Interaction of receptor-estrogen complex with specific acceptor sites on chromatin. Receptor estrogen complexes (RE) bind to two types of nuclear sites: nonacceptor sites (N) and acceptor sites (A). RE binding at A sites results in the stimulation of RNA synthesis which in turn leads to a stimulation of protein synthesis. For details see text.

(3, 24). These conclusions were derived from experiments in which nuclear accumulation and retention of the receptor was correlated with the extent of uterine growth (Fig. 4). The growth of the uterus 24 hours after an estradiol injection is maximally effected by 0.1  $\mu$ g of  $E_2$  (Fig 4). Maximal uterine growth correlates with the translocation of 10 to 20% of  $R_1$  to the nuclear fraction ( $\sim 2-4000$  sites/cell) and with the quantity of sites that undergo long-term retention in the nucleus (longer than 4-6 hours) (Fig. 4). Thus, the number of  $R_1$  sites (15-20,000/cell) exceeds the number required for maximal uterine growth and the retention of 10 to 20% of these sites in the nucleus for longer than 6 hours is correlated with maximal growth. The relationship between nuclear retention and uterine growth is also demonstrated in Fig. 5 (7). In these experiments, animals were injected with equal quantities of  $E_1$  or  $E_2$  and nuclear retention and uterine growth were measured at various intervals thereafter. The rapid accumulation of  $R_{n1}E_1$  and  $R_{n1}E_2$  by the nucleus correlates with the stimulation of early uterotrophic events and, as predicted from the number of complexes, the two hormones are of equal potency in this regard. However, the size of the uterus declines rapidly following  $E_1$  treatment, whereas  $E_2$  stimulates true uterine growth as reflected by in-

creases in dry weight and protein content. Thus  $R_nE_3$  complexes are cleared from the nuclear compartment within 6 hours while  $R_nE_2$  complexes remain elevated for some time.

These results demonstrate that the mere stimulation of early uterotrophic events does not lead to true uterine growth. It is clear that estriol is as efficacious as estradiol in the stimulation of early uterotrophic events, yet this compound does not produce significant true uterine growth as reflected by dry weight. This failure of estriol to produce true growth appears to be due to the loss of the  $R_nE_3$  complex from the nuclear compartment. Thus,  $R_nE_2$  complexes remain within the nucleus for longer periods of time than  $R_nE_1$  complexes, perhaps at a limited number of nuclear sites. This long-term retention may be necessary for the sustained

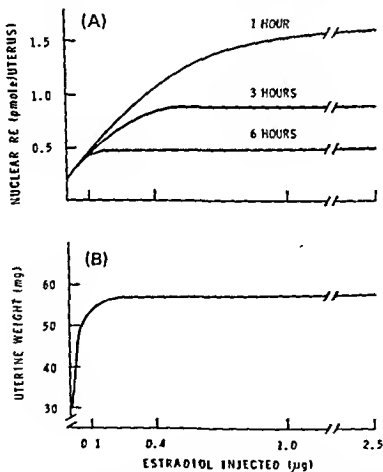


FIG. 4. Relationship between the amount of receptor-estrogen complex in the nucleus as a function of time and true uterine growth. Immature rats were injected with various quantities of estradiol and the amount of receptor-estrogen complex was determined in the nuclear fraction at 1, 3, and 6 hours after the injections (A). Twenty-four hours after the injections, the uterine weight was determined (B).

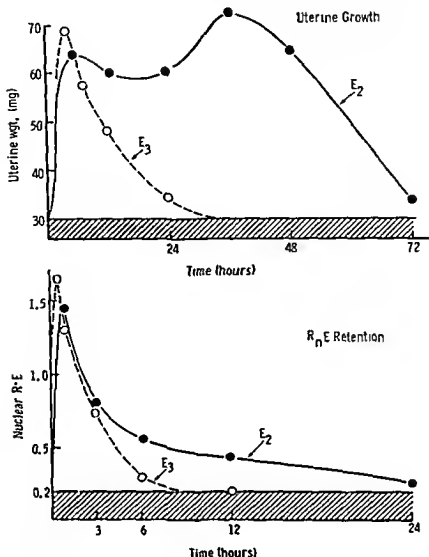


FIG. 5. Relationship between uterine growth and retention of the receptor-estrogen complex by the nucleus. Immature rats were injected with 10  $\mu$ g of the estradiol ( $E_2$ ) (●) or estriol ( $E_3$ ) (○) and the quantity of receptor-estrogen complex in the nucleus was determined at various times by  $^3H$ -estradiol exchange. Uterine weights were measured at the various times shown. The cross-hatched area represents the control levels of nuclear RE and uterine weights in saline injected controls.

stimulation of nuclear events that are important for the production of true uterine growth. The suggestion that long-term retention of hormones in tissues was necessary for full response has been made by other investigators (64, 112). Dimethylstilbestrol (DMS) and 16-oxoestradiol are "weak" estrogens and do not cause vaginal cornification after a single injection. However, repeated injections of these compounds does cause full vaginal cornification (64). We have shown that injection of estriol every 3 hours for 15 hours results in uterine growth that is equivalent to that produced by one injection of estradiol (7). Continuous exposure to estriol has also been shown to facilitate mammary tumor formation equally as well as estradiol (97). Thus continuous occupancy of receptors by an estrogen, regardless of its relative potency, will cause full biological

response. In the past,  $E_1$  has been called a "weak" or an "impeded" estrogen (47). It is clear from the above observations that this nomenclature needs revision. Instead of "weak," *short-acting* might be a more valid term for estrogen like DMS,  $E_2$ , mesestrol, and 16-oxoestradiol.

### C. NUCLEAR BINDING MECHANISMS

The precise interactions that occur when  $R_nS$  binds to sites on chromatin are not known. However, O'Malley and his co-workers (80) suggested that the  $R_nS$  complex binds to specific nonhistone proteins, NHP, and that these proteins represent acceptor sites (Fig. 6). The binding of the receptor progesterone complex to oviduct chromatin is dependent on the

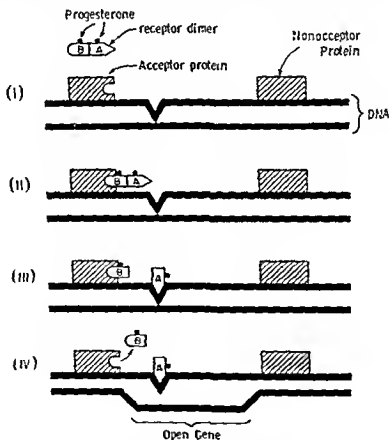


FIG. 6 Hypothetical scheme for gene activation by the receptor progesterone complex (I) The progesterone receptor is composed of two subunits, A and B. Each subunit binds a progesterone molecule (II) The receptor progesterone complex binds to an acceptor protein on DNA (III) The binding of the B subunit of the receptor progesterone complex in II results in a dissociation of the A subunit which is free to bind to specific sites on the DNA strand. (IV) The binding of subunit A to the binding DNA site results in a "melting" out of the DNA strands and opens the gene for transcription by RNA polymerase.

presence of these nonhistone proteins from oviduct nuclei, and not on histones (80). Furthermore, acceptor capacity could be transferred to nonoviduct chromatin by the oviductal NHP fraction (107). Therefore, it has been suggested that these nonhistone "acceptor" proteins might define specific sites on the genome with which the  $R_nS$  complex can bind and interact. The result of this specific binding would be the activation of appropriate genes (Fig. 6). Recently Puca *et al.* (88, 89) have isolated a protein fraction from uterine nuclei which binds the estrogen receptor in a specific manner. These sites are in excess of the number of "acceptor" sites as defined in Section IV,B and Fig. 3 and their relationship to these sites is presently not clear.

It has been suggested that steroid receptors are composed of two subunits, each of which binds steroid (100). The progesterone receptor in the chick oviduct is composed of two dissimilar subunits: subunit B, that binds to nonhistone chromosomal proteins (acceptor) discussed above, and subunit A, which binds to DNA (Fig. 6). In this model the A-B dimer steroid complex enters the nucleus and the B subunit recognizes and binds to acceptor sites on chromatin. These acceptor sites are located near the DNA binding site for the A subunit. The A subunit may dissociate and bind to DNA, presumably in a nucleotide sequence-specific manner. This binding interaction results in the activation of a specific gene. In this way the acceptor protein may designate appropriate genes and thereby direct the actions of the  $R_nS$  complex (101). These interactions and characteristics are summarized in Fig. 6.

The mechanism by which the  $R_nS$  complex is released or processed in the nucleus is not known and the changes which are shown in Fig. 1 ( $R_nS \rightarrow R_n'S \rightarrow S'$ ) are strictly speculative. Many other possibilities exist whereby the  $R_nS$  complex could be processed or deactivated, and these are under active investigation at the present time.

## V. Steroid-Induced Responses

Estrogen and progesterone influence the activity of many tissues and organs, however, none have been studied as extensively as the uterus and the chick oviduct. Therefore this portion of the chapter will be concerned only with the effects of estrogen on these two organs.

### A. EARLY UTEROTROPIC RESPONSES

Within the first 1 to 2 hours after an injection of estrogen many processes are activated in the uterus. These include histamine mobilization,

hyperemia, lysosome labilization, increased RNA, lipid, and protein synthesis, water imbibition, and increases in precursor uptake (Table III). Various investigators have proposed mechanisms by which these early events cause uterine growth. These proposed mechanisms invariably include a cascade mechanism by which a primary response sets off secondary and tertiary responses that ultimately culminate in the production of true uterine growth (10, 11, 109).

As pointed out earlier and as shown by several investigators, "weak" or short-acting estrogens like estriol will stimulate all early responses. However, single injections of these compounds do not cause true uterine growth (Table III). From these observations it can be concluded that a cascade phenomenon, which is stimulated by a primary event, is very

TABLE III

Comparison of Estrogen-Induced Responses by "Potent," Long-Acting Estrogens and "Weak," Short-Acting Estrogens\*

	Long acting		Short acting	
	Es-tradiol (E <sub>2</sub> )	Diethylstilbestrol (DES)	Estriol (E <sub>3</sub> )	Dimethylstilbestrol (DMS)
Early responses (1-6 hours)				
1. Histamine mobilization	+		+	+
2. Hyperemia	+	+	+	+
3. Wet weight increase	+	+	+	+
4. Increased glucose oxidation	+	+	+	
5. Increased lipid synthesis	+		+	
6. Synthesis of induced protein (IP)	+		+	
7. Early RNA polymerase activity (1-4 hours)	+	+	+	
8. Nuclear and nucleolar size changes	+		+	+
9. Increased precursor incorporation RNA and protein (1-4 hours)	+	+	+	
10. Increased initiation sites on chromatin	+		+	
Late responses (6-48 hours)				
1. Continued stimulation of RNA polymerase I and II	+		-	
2. Increase general protein synthesis	+	+	-	
3. Increase DNA synthesis	+	+	-	
4. Cellular hypertrophy and hyperplasia	+	+	-	-

\* These data have been taken from the work of Mueller *et al.* (74); Szego (109); Glasser *et al.* (37); Gorski *et al.* (40); Clark *et al.* (24); Anderson *et al.* (7); Katzenellenbogen and Gorski (55).



unlikely. Instead, these and other observations (see Section IV,B) substantiate the suggestion that long-term nuclear retention is correlated with the stimulation of true uterine growth (7). It appears that "weak" (short-acting) estrogens such as  $E_1$  and DMS do not possess the capacity to cause long-term nuclear retention of the receptors and, as a consequence of this deficiency, they fail to sustain the requisite responses which produce true growth.

The role of these early responses should not be minimized, however, because they undoubtedly play a very important role in the preparatory and supportive events that maximize conditions for uterine growth. An examination of the obligatory uterotrophic response pathways and how they relate to these early events is one of the most important areas of research in the field of estrogen action. As an example, the relationship of estrogen induced RNA polymerase activity to the stimulation of true uterine growth is discussed below.

## B STIMULATION OF RNA AND PROTEIN SYNTHESIS

Estrogen administration stimulates RNA and protein synthesis in the uterus. The earliest detectable increase occurs within 2 minutes and reaches a maximum by 20 to 30 minutes (44, 65). The activity then declines and remains low for 2 hours. This is followed by an elevation in activity which remains high for at least 24 hours (44). This early effect of estrogen on RNA synthesis is blocked by actinomycin D but not by cycloheximide (65). These data suggest that the effect of estrogen on RNA synthesis is direct and does not involve protein synthesis.

Recently several groups have reported an early, within 15 to 30 minutes, increase in very high molecular weight (DNA-like) RNA and have suggested that the marked increase in total RNA which follows may be dependent on this early appearance of mRNA (60). Complimentary to these results on high molecular weight RNA has been the observation that  $E_1$  treatment results in a comparably early increase in endogenous nuclear RNA polymerase II (transcribes mRNA) activity which is followed, after  $E_1$  administration (2-4 hours), by an increase in the activity of RNA polymerase I (transcribes rRNA) and a second rise in RNA polymerase II activity (13, 37).

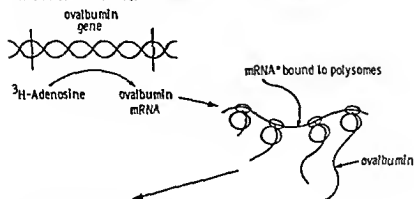
A number of laboratories have reported the stimulation of chromatin template capacity within short periods of time following estrogen administration (9, 37, 111). Chromatin template activity estimates the percentage of the total genome which is available for transcription by the endogenous nuclear RNA polymerases. Schwartz *et al* (102) and Tsai *et al* (114) have modified the template assay so that the number of initiation

sites for RNA polymerase binding can be determined. This assay has been used to demonstrate that estrogen causes marked increases within 30 minutes in the number of polymerase initiation sites on chick oviduct chromatin (54, 114 and see below). Similar studies have shown that estrogen also stimulates a rapid elevation in the number of initiation sites on uterine chromatin (S. Glasser, R. Schwartz, and J. Clark, unpublished). Therefore it is likely that during early estrogenic stimulation of the uterine genome the receptor estrogen complex may "open up" or make accessible more DNA sites for RNA polymerase. This proposal will be discussed further in Section V,C.

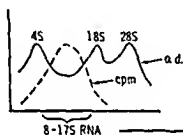
An increased number of initiation sites for polymerase binding and DNA transcription should produce specific mRNA's which are responsible for the translation of specific proteins. The model system that has proved most useful for the study of specific RNA and protein synthesis is the chick oviduct. This organ is undifferentiated and atrophic in the 7- and 8-day-old chicken. Administration of estrogen causes marked growth of the oviduct and the differentiation of three types of cells (79). The cell type that predominates is the tubular gland cell which makes large quantities of the egg white protein, ovalbumin (82). Therefore, the estrogen-treated oviduct should contain ovalbumin mRNA. In order to prove the existence of ovalbumin mRNA it was necessary to show that oviductal RNA would stimulate the synthesis of ovalbumin in a cell-free system for the translation of mRNA. For this purpose 8 S-17 S RNA from oviduct polysomes was extracted and used in the rabbit reticulocyte translation system (67, 93) as diagrammed in Fig. 7. It was possible to show that while no detectable ovalbumin mRNA exists in an unstimulated chick oviduct, estrogen administration caused marked increases in ovalbumin mRNA and that estrogen withdrawal results in a rapid decline in the quantity of mRNA (29). Indirect evidence that estrogen also stimulates increased synthesis of mRNA for glucose-6-phosphate dehydrogenase in the uterus has been provided by the work of Smith and Barker (105).

A more sensitive method for the detection of mRNA involves the use of  $^3\text{H}$ -DNA copies of the mRNA in question (12). Reverse transcriptase (a RNA-directed DNA polymerase) is used to make  $^3\text{H}$ -labeled DNA copies of purified ovalbumin mRNA, thus producing a labeled DNA copy of the mRNA. This  $^3\text{H}$ -DNA can then be used as a highly specific and very sensitive probe for the detection and quantification of ovalbumin mRNA. With this method the number of mRNA copies that are stimulated by estrogen has been calculated to be 15,000/cell. After withdrawal of estrogen this number declines dramatically to  $\sim 10/\text{cell}$ . From these elegant studies the evidence for the stimulation by estrogen of specific mRNA's appears to be very conclusive (81).

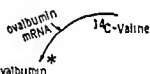
I Stimulation of Gene Transcription and mRNA Translation  
In DES Treated Oviduct



II RNA Extraction from polysomes  
and fractionation of RNA by  
Sucrose density gradient centrifugation



III Translation of mRNA  
in rabbit reticulocyte  
system



IV Identification of  
labeled ovalbumin  
by immunoprecipitation

FIG. 7. The identification and measurement of ovalbumin mRNA in estrogen-stimulated chick oviduct. The details of this experiment are described in the text.

The demonstration that progesterone operates in a similar fashion was more difficult because progesterone causes less dramatic changes in protein synthesis. In addition, progesterone causes no marked changes in RNA synthesis and the polysome profiles are unaltered (66, 79). Progesterone does stimulate the synthesis of an egg white protein, avidin. However, avidin constitutes ~0.1% of the total egg white protein (81). Thus, the detection of a progesterone-induced mRNA for avidin is extremely difficult when compared to the detection of the mRNA for ovalbumin. The discovery that some mRNA's have long polyadenylate residues at the 3'-terminal end of the chain and that these RNA's adsorb to nitrocellulose filters enabled investigation to detect and identify the mRNA for avidin (14, 95). With this method small quantities of avidin mRNA can be detected in the presence of large quantities of other RNA's. Avidin mRNA is present in the 8 S-17 S polysomal RNA fraction from the progesterone-treated oviduct and has a 9 S sedimentation coefficient. This value corresponds to

a mRNA that would code for a protein of approximately 15,000 daltons, the subunit size of avidin (20). A single injection of progesterone causes a significant increase in avidin mRNA by 6 hours and maximum concentrations are achieved by 18 hours (20). From the above it can be concluded that estrogen and progesterone stimulate the synthesis of specific mRNA's which are responsible for synthesis of new proteins which alter cell functions.

### C. RELATIONSHIP BETWEEN RNA POLYMERASE ACTIVITY AND UTERINE GROWTH

Estrogen causes growth in both the uterus and oviduct. Growth results from increased synthetic activity which produces cellular hypertrophy and hyperplasia (92). We have suggested that estrogen stimulates growth by virtue of its ability to form a hormone receptor complex which binds to specific acceptor sites on chromatin. This binding, which must be maintained for relatively long periods of time, appears to be involved with the stimulation of specific RNA and protein synthesis. The precise relationships between hormone receptor binding in the nucleus and nuclear events which produce true growth are not known. However, we have endeavored to explore these relationships by studying the differential effects of various estrogens and estrogen antagonists on RNA polymerase activity and uterine growth. In this manner we hope to dissect from the myriad of estrogen-induced responses those which are necessary, i.e., obligatory, for the production of true growth.

In these experiments estriol,  $E_1$ , and Nafoxidine, N (Upjohn 11,100-A), were used as examples of a short-acting "weak" estrogen and a long-acting "weak" estrogen, respectively, and compared with estradiol,  $E_2$ , a long-acting potent estrogen. All three compounds stimulate the activity of RNA polymerase II which reaches a peak at 1 hour and declines rapidly to control levels by 2 hours (Fig. 8). This activity is undoubtedly related to the early RNA synthesis that was discussed in the previous section. Estradiol and Nafoxidine treatment results in a second rise in polymerase II activity by 4 hours, which remains elevated for some time. However, this second increase in polymerase II activity is not seen in  $E_1$ -treated animals. Since  $E_1$  does not produce true uterine growth under these conditions, we conclude that this second rise in polymerase II activity is an obligatory step in the production of true growth (46). This rise may be responsible for the increased synthesis of these mRNA's required for cellular hypertrophy and hyperplasia. In addition to stimulating polymerase II, each of these hormones elevates the activity of polymerase I within 4 hours after injection.

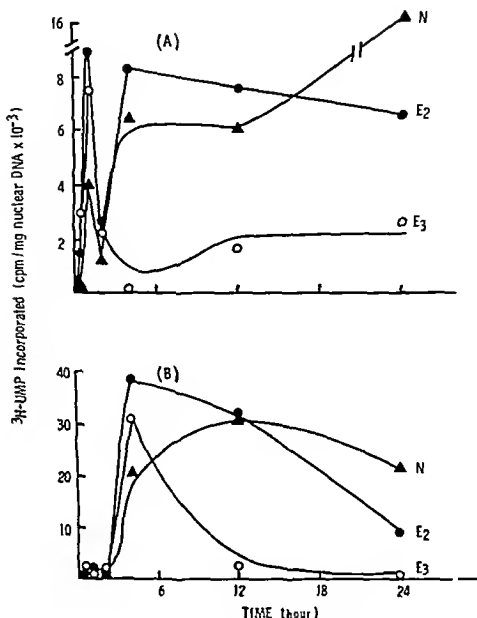


FIG 8 Time course of nuclear RNA polymerase I and II following hormone injections. Animals were injected with either estradiol, estriol, or Nafoxidine as described in the text. At indicated times, uteri were removed, nuclei isolated, and endogenous RNA polymerase activities determined. All points represent six to nine determinations. (A) RNA polymerase II activity; (B) RNA polymerase I activity. E<sub>2</sub>, ●—●; E<sub>3</sub>, ○—○; N, ▲—▲

tion. However, the effect of E<sub>3</sub> is transient and the enzyme activity fades rapidly (Fig. 8).

The failure of E<sub>3</sub> to stimulate a second rise in polymerase II activity or sustain polymerase I activity is correlated with the short-term residency of the R<sub>n</sub>E<sub>3</sub> complex in the nucleus (Fig. 5). On the other hand, treatment with E<sub>2</sub> or N results in long-term nuclear retention of the receptor by the nucleus (25). These corollaries and concomitant stimulation and sustained activity of polymerase I and II (46) can be extended in the case of E<sub>2</sub> and N. Nafoxidine treatment results in receptor retention for long

periods of time, up to 19 days in the immature rat uterus (25, 26), while the retention of  $R_nE_2$  is approximately 24 hours. As shown in Fig. 8, treatment with N results in sustained stimulation of both RNA polymerase activities to a much greater extent than  $E_2$ , which is probably related to its extended retention at nuclear sites. Proof of the relationship between nuclear retention and uterine growth can be demonstrated by administering  $E_2$  in a continuous manner, either by serial injection every 3 hours (7) or by pellet implant (J. Clark and E. Peck, unpublished). Under these conditions,  $E_2$  is a potent estrogen, producing long-term continuous retention of the receptor by the nucleus and resulting in marked uterine growth.

These experiments together with those in the preceding sections, suggest that the receptor hormone complex,  $R_nE$ , binds to and is retained at specific nuclear sites. The initial binding results in increased template activity and opening up of gene sites, which results in the early peak in polymerase II activity. The products of this burst of RNA transcription are probably involved in but not the primary cause of true uterine growth. True uterine growth is seen only with a second elevation in polymerase II, as observed for  $E_2$  and N, but not for  $E_2$ . The continued stimulation via long-term retention of the  $R_nE$  complex causes the sustained elevation of polymerase I activity required for the enormous increases in ribosomal RNA synthesis. Thus, the stimulation of the genome by the  $R_nE$  complex produces large increases in all classes of RNA which are involved in the qualitative and quantitative changes in protein synthesis observed when uterine cells grow.

The template activity of chromatin increases rapidly following estrogen administration and, as discussed in the previous sections, this increase is associated with a rapid elevation in RNA polymerase II activity. These activities are assumed to be associated with an increased number of polymerase initiation sites on chromatin. However, they could be due to elongation of existing RNA chains, reinitiation of existing sites, and/or increased number of polymerase molecules in an active state. These initiation sites can be measured and it has been shown that estrogen treatment causes dramatic increases in the number of polymerase initiation sites on oviduct chromatin. In these experiments the quantity of receptor in the oviduct nucleus increases dramatically after an injection of DES and this precedes the stimulation of RNA polymerase initiation sites (54). Both of these events precede the appearance of ovalbumin mRNA, thus establishing the appropriate temporal sequence of events (114). The relationships between the number of receptor binding sites in the nucleus and the number of initiation sites for RNA polymerase is not clear at the present time, however we have been able to show close correlations between these parameters during growth of the oviduct and during regression following hormone removal (54).

The results of these experiments indicate that the receptor-steroid complex binds to specific sites on chromatin and that this binding results in the opening up of initiation sites for RNA polymerase. This could be accomplished through a mechanism by which the binding of the  $R_E$  complex partially or totally melts out the DNA of that region which, in turn, opens the DNA for binding by polymerase molecules (Fig. 9). The polymerase molecule then transcribes the DNA (gene) and produces new and/or more RNA which in turn is responsible for increased protein synthesis. These proteins are involved in altering and controlling cell function and growth or may be secretory products of the tissue. However, as pointed out earlier, the mere act of "turning the genome on" is not sufficient to cause true growth of the uterus. Therefore, in any model of steroid induction of growth, a nuclear residency factor must be considered.

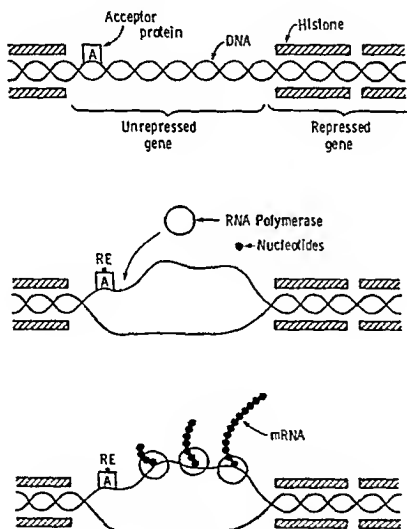


FIG. 9. Gene activation and estrogen receptor binding. A receptor-estrogen complex (RE) binds to an acceptor site (A) which is bound to DNA. This interaction results in a "melting out" of the adjacent DNA region (gene) which opens up the DNA for RNA polymerase binding. The polymerase molecules are then free to transcribe the DNA into RNA.

## VI. Interactions between Steroid Hormone Receptors

In the previous sections of this chapter the action of each hormone was considered separately. However, it is clear that hormones interact to modify and sometimes antagonize each other. These interactions have been recognized at the physiological level for many years, but only recently have the mechanisms of these interactions begun to unravel.

### A. ESTROGEN AND ITS CONTROL OF THE PROGESTERONE RECEPTOR

The uterus is relatively insensitive to progesterone unless it has been exposed to estrogen.\* Thus, progesterone treatment in a nonestrogenized uterus will not produce a secretory uterine epithelium (92); however, with estrogen priming progesterone has dramatic effects on the production of secretory responses. Since it is generally agreed that progesterone acts by binding with a specific receptor (see Section III,B), these observations may be explained *a priori* by assuming that estrogen priming stimulates the synthesis of the progesterone receptor, thereby enhancing the ability of the uterus to respond to progesterone (30, 33, 34, 48, 71, 72, 90). Leavitt *et al.* (61) demonstrated clearly that estrogen was required for the maintenance of the cytoplasmic progesterone receptor in the hamster uterus. In these studies, hamsters were ovariectomized at proestrus and the quantity of cytosol progesterone receptor measured as a function of time (see Fig. 10). The quantity of receptor fell dramatically and reached low levels by 2 weeks. Estrogen treatment at this time caused a marked elevation to proestrus levels within 24 hours. From these observations it can be concluded that estrogen stimulates the uterus to produce more cytosol progesterone receptor, probably by *de novo* synthesis. Thus, estrogen sets the stage for the binding of progesterone which is a prerequisite for progesterone action.

### B. CONTROL OF THE ESTROGEN RECEPTOR BY PROGESTERONE

Progesterone has long been considered an antagonist of estrogen action (62). The delicate balance and interactions between these ovarian hormones are essential for many reproductive functions. Earlier studies in the chick oviduct and rodent uterus have shown that the simultaneous administration of progesterone and estrogen resulted in inhibition or modification

\* A major exception to this statement is the observation by Glasser (38) that the decidual response can be produced in rats which have received only progesterone.



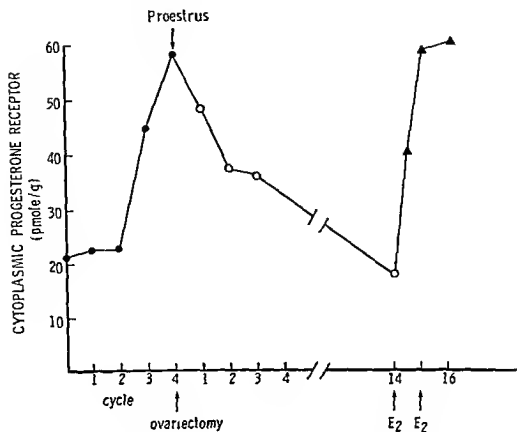


FIG. 10 Effect of ovariectomy at proestrus on the concentration of uterine progesterone binding sites. Results for the cycle (●—●) are provided for comparison. The concentration of binding sites declined more slowly after ovariectomy at proestrus (○—○) than between proestrus (P) and estrus (E) in intact animals. Estradiol-17 $\beta$  treatment ( $E_2 = 15 \mu\text{g/day}$ ) initiated 14 days after ovariectomy restored the concentration of progesterone binding sites to the proestrus level (▲—▲).

of estrogen-induced growth of these target organs (16, 66, 78). Since the source of progesterone antagonism of estrogen action is likely to involve estrogen receptor function, the effects of progesterone on the estrogen receptor have been the subject of recent investigations. Neither initial binding of estrogen to the cytoplasmic receptor,  $R_c$ , nor the translocation of the  $R_cE$  complex to the nucleus is inhibited by progesterone (2, 113). Hence the source of its antagonistic effect does not lie at these levels.

One of the ways that progesterone could antagonize or modify estrogen effects is by interfering with receptor availability, i.e., reduced  $R_c$  synthesis or reutilization. This mechanism has been suggested by several laboratories (15, 63, 69). Recent work from our laboratory indicates that progesterone interferes with the replenishment of the cytoplasmic estrogen receptor and this reduction of  $R_c$  is correlated with a diminished or modified sensitivity of the uterus to estrogen (49, 50, 68). As shown in Fig 12, cytoplasmic receptor replenishment appears to involve two phases. The first phase, 4–8 hours after estradiol injection, may represent a recycling of  $R_c$  which is not blocked by progesterone (Fig 11). The quantity of  $R_c$  that is

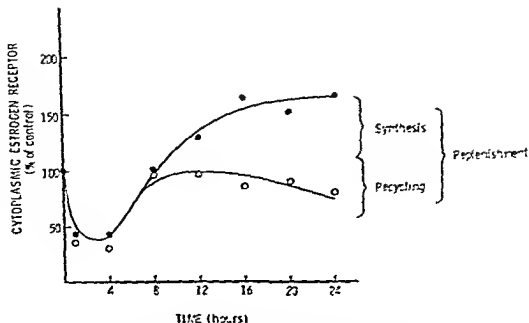


FIG. 11. The antagonism of estrogen receptor replenishment by progesterone. Estrogen-primed immature rats received either estradiol (●) or estradiol plus progesterone (○) at time zero. The quantity of cytoplasmic estrogen receptor was measured by the exchange assay at various times after injection.

replenished is equal to that which was depleted, suggesting that recycling through some deactivation-reactivation mechanism could exist and account for this phase of replenishment. The second phase, 8–24 hours after injection, may involve the synthesis of  $R_e$ , which is blocked by progesterone treatment. The quantity of  $R_e$  that is replenished during phase two is approximately 50% greater than the quantity of  $R_e$  which was initially present so that after both phases  $R_e$  is approximately 150% of control. These data suggest that  $R_e$  replenishment in estrogen-primed animals may be accomplished by two mechanisms: (1) a recycling of existing  $R$  following nuclear accumulation, which is not blocked by the presence of progesterone; and (2) a synthesis of new  $R$ , which is blocked by progesterone. A recycling process for glucocorticoid receptors that does not involve RNA and protein synthesis has been proposed by other investigators (70, 96). The involvement of protein synthesis in the replenishment process has been suggested by Gorski (39) and Cidlowski and Muldoon (21). Recently, Mester and Baulieu (68) suggested that the replenishment of  $R_e$  in uteri of immature rats involves two separate processes. The first process, 0–6 hours after injection, could not be blocked by cycloheximide, whereas the second process, 6–11 hours after injection, was dependent on protein synthesis. Thus, in cell types which grow in size or number in response to a steroid hormone,  $R_e$  replenishment may involve both recycling and synthesis of

new receptor, whereas in cells that do not grow in response to their target steroid,  $R_c$  replenishment may involve only recycling. The possibility that  $R_c$  replenishment is accomplished in some cell types by recycling and in others by synthesis cannot be ruled out. However, this seems unlikely since progesterone blocks the estrogen-induced replenishment of  $R_c$  in both the endometrium and myometrium (Hsueh *et al.*, unpublished).

The reduction of estrogen  $R_c$  by progesterone does not appear to be the only mechanism by which progesterone can modify estrogen action. Progesterone also reduces the nuclear retention time of the  $R_nE$  complex (50). As explained earlier (see Section IV,B) a reduction in nuclear retention time would decrease the ability of estrogen to stimulate uterine growth, and endometrium and myometrium (Hsueh *et al.*, unpublished).

The interplay between estrogen and progesterone may be considered in the following way (Fig. 12). Estrogen, E, binds to the cytoplasmic estrogen receptor,  $R_c^E$ , to form a complex  $R_c^E E$  which translocates to the nucleus,  $R_n^E E$ . This complex is responsible for the stimulation of events which lead to an increased quantity of  $R_c^E$  and of the cytoplasmic progesterone receptor,  $R_c^P$  (28, 61, 71). Estrogen also stimulates uterine hypertrophy and hyperplasia (Fig. 12,I). The elevated levels of  $R_c^P$  augments the ability of the uterus to respond to progesterone (Fig. 12,II) (8, 48, 118). Progesterone binds to its cytoplasmic receptor,  $R_c^P$ , undergoes translocation to the nucleus,  $R_n^P$ , and elicits the characteristic progestational responses that prepare the uterus

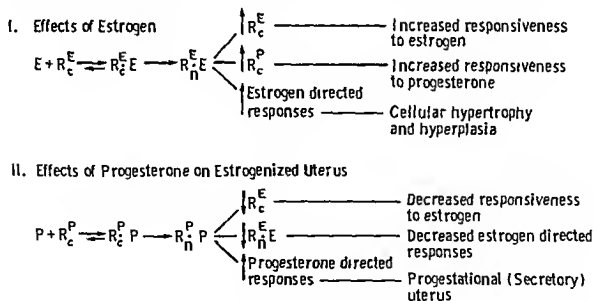


FIG 12 Interaction of estrogen and progesterone in the control of receptor levels and uterine growth. E, estrogen;  $R_c^E$ , cytoplasmic estrogen receptor;  $R_c^E E$ , receptor-estrogen complex in the cytoplasm;  $R_n^E E$ , receptor-estrogen complex in the nucleus; P, progesterone;  $R_c^P$ , cytoplasmic progesterone receptor;  $R_n^P P$ , receptor-progesterone complex in the nucleus (See text for details)

for implantation. Progesterone also reduces the availability of the estrogen  $R_E$  and the nuclear retention time for  $R_E$  thereby decreases the ability of the uterus to respond in a totally estrogen-directed fashion (50). Thus progesterone reduces and/or redirects the ability of the uterine cells to respond to estrogen. This interaction produces an appropriate uterine environment for implantation and pregnancy.

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# 7 Oogenesis and Folliculogenesis

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## 1. General Characteristics of Female Gametogenesis

The entire cycle of gametogenesis in the mammalian female results in ovulation when the oocyte is shed after extrusion of the first polar body and the first maturation division is complete. Female gametogenesis is not a continuous process like spermatogenesis. The oocyte pauses during meiotic prophase at the diplotene stage and only resumes development after transformations that characterize the female gamete, namely increase in size and build up of reserves. However, the oocyte passes through all the classic phases of gametogenesis: multiplication of germinal cells, preparation for haploidization (heterotypic prophase, gamete differentiation), and finally meiosis.

In terms of ovarian morphology, these processes are oogonial multiplication, formation of oocytes and primordial follicles, folliculogenesis and growth of the oocyte, maturation of the oocyte, and ovulation.

Oogenesis is a process which includes all these stages, the term "oogenetic period" is applied to the formation of the oocyte at the diplotene



stage. The period that follows is particularly important for the follicle, and is called "folliculogenesis."

## II. Formation of Primordial Follicles: Oogenesis

### A. ORIGIN OF PRIMORDIAL GONOCYTES

#### 1. Characteristics of the Primordial Germ Cells

The primordial germ cells of mammals possess histological characteristics which make them easily recognizable in certain species, particularly in man (46). The nuclei of these cells are large and weakly staining like those of large blood-forming cells (171). Their cytoplasm does not have the high concentration of vitellus that facilitates their identification in other vertebrates.

Among the histochemical characteristics [cytoplasmic PAS reaction (148), basophilia with alkaline toluidine blue affinity (161)], the alkaline phosphatase cytoplasmic staining revealed by the azodic technique has been most widely applied as described in man (89), mouse (26), rat (117), cattle (71), rabbit (28), and sheep (108). This cytoplasmic activity is asymetrically distributed (71) as a spot in proximity to the nucleus (108). The primordial germ cells possess a large Golgi complex (39) with high phosphatase activity (68) which could be related to this black photonic localization. This activity is also present in the cellular membranes (68). However, these are not the only embryonic cells (126) to possess alkaline phosphatase activity, therefore, this cannot be taken as an absolute criterion.

The ultrastructural characteristics of the primordial germ cell are more precise than the histochemical ones. A high electron cytoplasmic density results from the large number of free ribosomes and polysomal arrays associated with basophilia. There is a poor endoplasmic reticulum in contrast to somatic cells (161). During the migratory period of these cells, junctional complexes with somatic cells precede the presence of cytoplasmic bridges frequently described in oogonia (174).

#### 2. Experimental Evidence for an Extragonadal Origin of Primordial Germ Cells

The alkaline phosphatase positive cells are the same cells described as gonocytes in man by Witschi (171). They become primordial gonocytes in the differentiated gonad of both sexes as shown by their persistent

phosphatase activity. Experimental proof was also presented by Everett (47) and Mintz (110, 113). Transplantation of the embryonic genital ridges under the renal capsule of an adult, before this epithelium is populated by migratory cells, does not lead to the formation of any germinal elements; this formation is produced only if the graft is performed when migration is already complete (47). Moreover, in mouse embryos homozygous for mutant alleles at locus *W*, the number of primordial germ cells is normal at the eighth day of gestation, but becomes smaller or nil by the tenth day (112). Mice with such genotypes have few or no oocytes in their ovaries at birth (106, 113).

### 3. Gonad Settlement

The scheme described by Zamboni and Merchants (174) in mice has general value, and the dominant localization of the primordial germ cells are, respectively, hind gut, dorsal mesentery, and undifferentiated gonad. The chronological steps are summarized for different mammals in Table I and illustrated for sheep in Plate 1. In the chick embryo, blood vessels act as the normal migratory pathway from the germinal crescent to the gonads (157), but this is not the case in cattle (71) or sheep (108).

In birds, there is, in succession, passive transport, amoeboid displacement with chemotaxis, and unspecific attraction (38). The germinative epithelial cells have been shown to induce attractive displacement related to their glycoprotein biosynthesis (30, 40). In mammals, a similar phenomenon would explain the selective localization of the primordial germ cells under this epithelium at the end of the migratory phase.

TABLE I

Chronological Steps of Gonadal Settlement and Differentiation as Studied by Alkaline Phosphatase Method

Criteria	Species*				
	Mouse (1)	Rat (2)	Rabbit (3)	Cow (4)	Sheep (2)
First germ cells identification	8	11½	9	25	17
Germinative crests appearance	10	11¾	10-12	30	22
Absence of extragonadic germ cells	13	15½	16-18	34	34
First seminiferous cords	12.5	14½	14.5-15.5	40-42	35

\* Key to references. (1) Mouse (110, 111-112, 113); (2) rat and sheep (108), (3) rabbit (28), (4) cow (71)

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## B SEX DIFFERENTIATION OF THE GONADS AND GENITAL TRACT

## Morphological Differentiation of the Gonads

Classically, it is proposed that two proliferations of the superficial epithelium of the undifferentiated gonad invade the underlying mesenchyme. If the embryo is genetically male, the initial proliferation of the epithelium provides the sexual cords which differentiate into seminiferous tubules, and there is no further proliferation. In the female, the first proliferation provides the sex cords which will form the ovarian medulla. The second proliferation gives rise to the ovarian cortex which is separated from the medulla by a layer of connective tissue.

A recent theory is that the female fetus has no differentiated ovaries before the appearance of the first primordial follicle (72). It is certain that males differentiate very early and females are determined by the absence of differentiation. In the male, sexual differentiation of the gonad is characterized by a round macroscopic shape, an outer conjunctive zona, and the presence of seminiferous cords. These events appear in the rat at 14-25 days (72), in the calf at 39 to 40 days (70), and in sheep at 34 to 35 days (50, 99, 141). Before these structural modifications occur, an incipient germ cell scarcity at the surface of the male primordium testis contrasts with a preferential localization of germ cells in the outer zone of the female gonad at 32 days in the sheep (108). In the rat, cells with a clear loose cytoplasm encompass germ cells at 13 days and 9 hours (70). The relationship between germ cells and somatic cells seems to be a determinant of sexual differentiation.

## C COURSE OF OOGENESIS

Oocytes are developed in an identical pattern for all species (Fig. 1). Their development can be divided into three stages: oogonial, mitotic,

PLATE 1 Germ cell settlement and sexual differentiation of the sheep embryo gonads as revealed by alkaline phosphatase reaction

1 *Embryo at 18 days*: the germ cells are present along the margin of mesonephros ( $\times 230$ )

2 *Embryo at 23 days*: the germinal crests are differentiated. Their settlement by the primordial germ cells is in progress on the ridge ( $\times 230$ )

3 *Female embryo at 32 days*: the peripheral localization persists. An intense alkaline phosphatase activity is developing in the medulla ( $\times 90$ )

4 *Male embryo at 32 days*: the scarcity of the cortical zone in germ cells becomes evident. The interstitial tissue between sexual cords is highly alkaline phosphatase activated ( $\times 90$ )

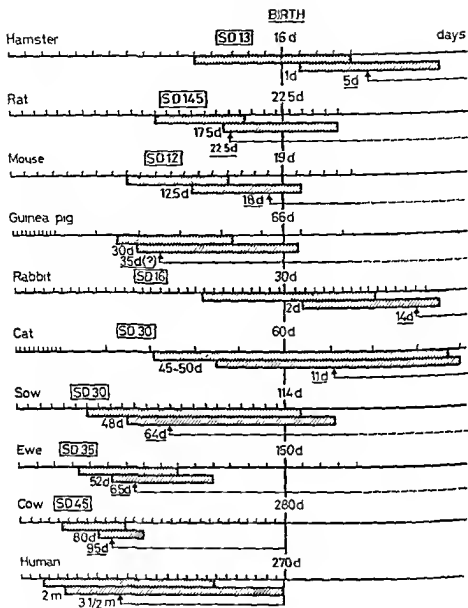


FIG 1 Comparative development of oogenesis in mammals. d, Days; m, months; SD, sexual differentiation. The first number gives the age of the first heterotypic prophase stages, the second, underlined number corresponds to the age of the first diplotene stages. The dotted strip represents the period of oögonial mitoses and the hatched strip shows the period when first meiotic prophase figures are present. The arrows point out the beginning of degenerative phenomena. See references (25 and 191).

divisions; meiotic prophase; somatic cell organization around diplotene oocytes, i.e., primordial follicle construction.

Great differences exist in the length of the multiplication period of germ cells. In the mouse, rat, and rabbit, mitoses are very rare after the appearance of first meiotic prophase (11, 17, 27). However, in the guinea

pig, ewe, cow, sow, monkey, and in the human female, these divisions are numerous even after the first meiotic prophase, and persist for a long time after the appearance of the first primordial follicles (4, 5, 43, 67, 100). The quantitative production of female gametes appears to be related to oogonial divisions.

However, intense degenerative phenomena in the germ cells have been observed in all species, particularly in the cow, cat, and ewe (99, 170). This degeneration can effect oogonial mitoses and oocytes at the pachytene or diplotene stage (4, 11). During oogenesis, in the midst of the isogenic groups, the cells that are retarded degenerate (11), but after oogenesis, degeneration occurs among the earliest oocytes formed, i.e., those lying deepest in the cortical zone (78).

Very little is known about this phenomenon, which plays such an important part in determining the number of oocytes remaining after oogenesis; many authors have, in fact, been struck by its importance and have even hypothesized about the possibility of there being a different mode of formation of female gametes (170).

## 1. Arrest or Persistence of Oogenesis in the Adult

It is now possible to confirm that the primordial follicles formed during the oogenetic period are "held in reserve" in the ovary and that their number does not increase further as shown by DNA labeling during oogonial mitosis (151, 152).

## 2. Variations in the Length of Oogenetic Period

Figure 1 shows the great variations which occur in the length of the oogenetic period between species. Oocyte labeling according to the age of embryo at the day of injection of tritiated thymidine demonstrates the oogenetic period from which the oocyte reserve is built up (Fig. 2).

In the rat, 85–90% of this reserve comes from oocytes entering into meiotic prophase on the 17.5th, 18.5th, and 19.5th day of embryonic life (101, 106). In the ewe, oocytes in preleptotene stages, as well as at the beginning, the middle, or the end of a long oogenetic period (52nd to 82nd day of embryonic life) are concerned with the constitution of the hundredth day postcoitus (p.c.) ovarian stock (106). However, a more active part in this constitution is played by the 52nd to 64th day p.c. period. In the pig this equivalent period is the 70th day p.c. (13).

The equivalence in quality of the gametes with their production time is related to the reserve utilization during folliculogenesis. If the selection of follicles for growth is nonrandom (41), the last formed oocytes should be

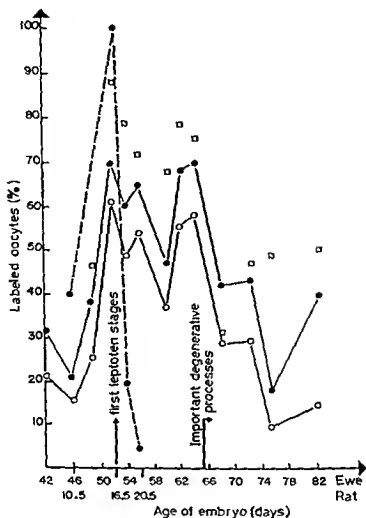


FIG. 2. Percentage of labeled oocytes related to the day of the tritiated thymidine injection in the ovary of the 100-day sheep embryo (uninterrupted line) and in the ovary of the 20-day postnatal rat (dotted line). For the sheep embryo, the three curves have the same shape whatever the method of labeling determination: microphotometer Leitz (unfilled square), silver grain counts with three grains (solid circle), or four grains (unfilled circle) as background value (106, 107).

the most defective. Indeed, in very old mice, oocytes present a diminishing chiasmata frequency, a change in chiasmatic chromosome localization, and an increase in univalent frequency (60).

### 3. Origin of the Variations in the Definitive Stock of Oocytes

The number of oocytes is greatest before the end of the period of their formation: 110 to 130th day p.c. in cattle (42, 43), 50 to 90th day p.c. in pig (13), 18.5–19.5th day p.c. in the rat (10), 45th day p.c. in the guinea pig (67), fifth to sixth month of the embryonic life in human females and

in the rhesus monkey (5, 6), and 17th day of egg incubation in chicks (64).

Degenerative phenomena produce a sharp drop in the number of oocytes in all species when the primordial follicles appear, i.e., the arrest of the meiotic process.

The oocyte count falls off rapidly as the animal grows older (3, 15, 16, 66, 80, 85, 159, 167); in the rat, mouse, and in the human female, the follicles can even totally disappear from the ovary before death. The fall in the absolute number of follicles is sharpest at first: 50–60% disappear during the first weeks after birth in the rat and mouse (69) or during the first year in guinea pigs (67). The proportion of oocytes lost per unit time is constant (81). However, this decrease in the reserve of primordial follicles is difficult to demonstrate with certainty in the few months after birth in the ewe, and in the cow it only becomes apparent after the fourth year (42, 43). At a given age, this reserve appears to vary between individuals of the same species. The ratio of the numerical extremes ranges from 1:2 in the mouse (69) and the rat (102), 1:3 in the ewe, and 1:10 in the cow (42, 43, 101).

In mice (69) and rats (84, 104) at birth, ovarian reserves of primordial follicles differ significantly between strains and F<sub>1</sub> crosses, i.e., at the end of oogenesis. These differences are maintained during the postnatal shift of oocyte counts (69, 102, 104).

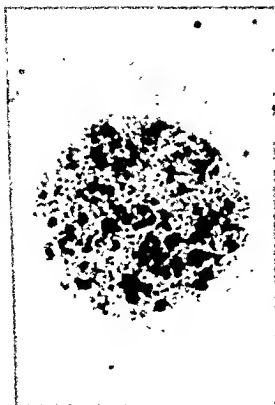
Such genetic factors modifying oocyte production could be related to germ cell multiplication during their migration as described in mice bearing the *W* gene (113). In rats, strain differences in the number of germ cells appear at 13.25 days of embryonic life (104), increase at 14.25 and 15.25 days, and reach a maximum at 18.25 days.

Some adult gonads have no germ cells, such as freemartin cattle, mule or hinny, and XO women. In all these cases, oogonia are present with their subsequent mitotic divisions in the 70–77th day fetal freemartin gonad (72, 73), in the 60-day mule fetus (111), or in the 3-month human fetus (158). Some germ cells enter meiosis (72, 73, 163), but due to the inability of the paternal and maternal sets of chromosomes to form homologous pairs (163) or to an inhibiting action of somatic cells as in XX/XY chimeras (90), the beginning of meiotic prophase is disrupted. The inhibitory action of somatic cells could be also a factor in quantitative female gametogenesis.

a. GERM CELLS AND OOGONIAL MITOTIC DIVISIONS. In the male, a germinal cell becomes a primordial gonocyte when the gonad is differentiated. In the female, the primordial germinal cell becomes an oogonium when the gonad differentiates into an ovary. However, does this cell



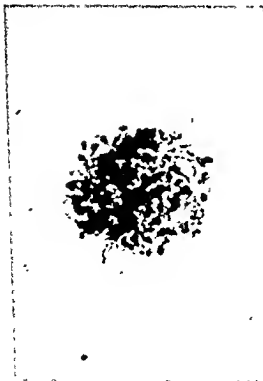
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possess the particular characteristics which distinguish it from the male gonocyte? In both sexes they divide mitotically during their migration (26, 27, 71, 111, 171). The germ cell mitotic activity stops at the preleptotene stage in the female gonad, and continues in the male gonocytes (9, 28).

*i. Morphological types of oögonia.* In addition to the stages of meiotic prophase, Winiwarter (169) distinguished three nuclear types in the rabbit ovary: protobrochal a, protobrochal b, and deutobrochal nuclei. After a study of the cat ovary, a new type was added, termed "dusty" by analogy with the "dusty" spermatogonia described by Regaud (144). According to Winiwarter, only the "protobrochal" types correspond to oögonia. The deutobrochal type with its reticulate nuclear aspect looks like a stage of the preleptotene figures (88). In human (162), rabbit (37), and sheep embryos (109), a developmental condensation of chromosomes followed by decondensation is described (Plate 2) similar to that reported by Walters (168) in *Lilium*. These stages would represent a true mitotic reversion in which metaphase and anaphase would be omitted (12).

*ii. Scheme of oögonial division.* By germ cells counts in a great number of rat embryonic gonads with age differences of a quarter of a day divisions were found at 11.75, 12.50, 13.25, 13.75, and 14.75 days of the embryonic life regardless of sex. The last two divisions were confirmed by the evolution of the intensity of oocyte labeling according to the day of tritiated thymidine injection. This method added a 15.75 day division (101). An oögonium undergoes a mean of six divisions from 11.25 days of age. Since the curve of the evolution of the number of cells is exponential in the rat, each germ cell must be submitted to a similar number of divisions.

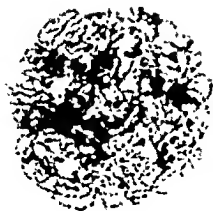
Synchronized phenomena can be observed in terms of space and time, but an asynchronism of the mitotic or meiotic activities are evident from the individual differences in germ cell counts in the same aged embryos or in the first appearance of meiotic figures between animals of different ages. Such an asynchronism is limited to 1 day in the rat (may be two in the ewe), and expresses the variable delayed phenomena occurring in some cells of the same ovary. It can be termed as the existence of "waves."

In the ewe, "waves" do not explain the shape of the curves of the per-

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PLATE 2. Cytological figures of the preleptotene stages in the 60-day sheep embryo ( $\times 2500$ ). 1. Early maximal chromosome condensation (stage II). 2. Maximal chromosome condensation (stage III). 3. Polarization and early chromosome decondensation (stage IV). 4. Chromosome decondensation (stage V). Stages refer to usage of Mauleon *et al.* (109). Orcein-lactic-acetic staining after alcohol-acetic acid fixation described in reference (109).

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centage of labeled oocytes (106, 108) or blocked oogonial mitotic number (99). Perhaps a renewal of undifferentiated oogonia gives rise to such a long survival of oogenetic processes. Then, two or three similar generations of oogonial divisions, as described in the rat, could be intermingled in the ewe (106).

b. MEIOTIC PROPHASE. Winiwarter (169), refers to Van Beneden's work on oogenesis in *Ascaris*, and gives a precise definition of the whole series of nuclear modifications of the meiotic prophase. His terminology for the different stages is still used today: leptotene, zygotene, pachytene, diplotene. These stages differ very little from one species to another (Plate 3).

i. *Variations in chromosomal configuration at the dictyate stage and radiosensitivity.* The oocytes are arrested at the same stage of meiotic prophase, termed "dictyate," but the chromosomal configuration of this stage of arrest varies among species. It could permit the identification of ovaries of many species (human, mouse, guinea pig, goat, and dog) (121). In cattle, this arrested stage of oogenesis may be the pachytene stage (61, 143). However, this stage very much resembles the so-called contracted stage observed in the guinea pig, and may, as in the guinea pig, be a modified diplotene stage (67). It is more frequent than the stage in which the chromosomes are distributed over the whole nuclear area, corresponding to the diplotene stage (165). This is the best explanation for the higher radioresistance of the oocyte of the primordial follicle in cattle (42), since the pachytene stage of meiotic prophase is the most sensitive to X rays (9, 10, 92, 130, 132).

The differences between species in the radiosensitivity of primordial oocytes arise from these variations in chromosomal configuration at the dictyate stage (5, 92, 121). It has been shown that oocytes with lampbrush chromosomal filaments are particularly radioresistant. This lampbrush structure is found in the oocytes of human primordial follicles (6) and in the follicle at the start of its growth in the rat (5). In the same way, the "contracted" structure of the guinea pig oocyte corresponds to a higher radioresistance than the classic diplotene stage of the mouse oocytes (121).

ii. *Duration of meiotic prophase.* The time intervals between the appearance of the first oocytes at leptotene stage and the first oocytes at

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PLATE 3. Cytological figures of meiotic prophase in the 60-day sheep embryo ( $\times 2500$ ). 1. Leptotene; 2. Zygotene; 3. Pachytene; 4. Early diplotene. Orcein-lactic-acetic staining after alcohol-acetic acid fixation described in reference (109).

diplotene stage differ in females of different species: in the rat, mouse, and hamster, the interval is around 4 to 5 days (11, 25), in domestic mammals it is from 10 to 15 days (99), and in the human it is approximately a month (8).

Using radiotracers, the progression of labeling gives similar durations of the leptotene (3–8 hours) and zygotene (12–40 hours) stages in the mouse (29), rabbit (79), and ewe. The pachytene stage lasts 60 hours in the mouse, 6–9 days in the rabbit, and 10 days in the ewe.

The arrest of meiotic prophase after the diplotene stage is due to a nonhomologous association of bivalent chromosomes during oogenesis (123, mouse; 125, cattle). It is frequently observed in the oocytes, and only occasionally during prophase of somatic cells or during the pachytene stage of spermatogenesis. According to Ohno, it is the persistence of this association at the diplotene stage that leads the oocytes to the dictyate stage instead of their undergoing diakinesis and degeneration (127). In a 17- to 20-day mouse fetus, when intense degeneration exists, the diplotene stage without nonhomologous association of bivalents is observed. Somatic cells of a 8- to 12-day chicken female gonad must be organized conveniently around the germ cells for their normal oogenetic evolution *in vitro* (75).

iii. *Role of the rete ovarii in the onset of meiosis and primordial follicle organization.* The role of the rete ovarii has been recently demonstrated (22). Within the ovaries of the mouse, cat, ferret, and mink, at the time meiosis begins, rete cells are secretory and an open connection between the rete system and the germ cells exists (24). The diplotene arrest, i.e., the maintenance of dissociated bivalents, could depend upon the surrounding somatic cells (125). Byskov's results suggest that the rete cells are also essential in the organization and early formation of the follicles (131).

iv. *Meiotic inducers and dependence of oogenesis on hormonal factors.* Oogenesis can continue if the ovary is cultured *in vitro* (8, 98, 105, 154). However, meiosis in the germ cells of the embryonic ovaries does not normally occur in organ culture when the ovary is removed before a critical period: 50th day in the sheep embryo (105, 108) and 13 to 14th day in the hamster fetus (25). A cytological and histological study of gonads that have been cultured for 6- to 12-day periods shows that at meiosis, preleptotene and a few abnormal zygotene figures are developing (105, 108). The dissection of the mesonephric part as suggested by Byskov's results (22) does not change the previous observation, perhaps because the rete cells are already present in the medullary portion of a 28-day sheep

embryonic ovary (74). In the same culture conditions, the meiotic processes even appear if the ovary is removed at 13 days p.c. in the rat (109).

In sheep, the germ cells during their *in vitro* period retain their potential to initiate the chain reactions that induce meiosis since the cultured ovary (108) leads to a supposed delay in the resumption of meiosis after grafting. In spite of specific differences, the onset of meiosis can be modified by the following conditions:

1. A necessary change in oogonia development for a further *in vitro* induction and evolution of meiosis.

2. A local favorable environment for such an induction as shown by Byskov's studies that would explain the rare pachytene stages reached by the XX germ cells in the XX/XY chimeras (90) if the presence of XX somatic cells becomes necessary.

3. A possible diffusible inducing substance present in the developing ovary as suggested by the onset of meiosis in the germ cells of an 8-day chicken embryo male gonad cultured with a 12-day female gonad (74). The meiotic induction in isolated germ cells cultured on a cortex fragment also favors such a hypothesis.

4. A meiotic inhibiting substance developed perhaps after male sexual differentiation. With *in vitro* culture, the differentiation of the ovary is slightly retarded by the presence of a testis. Structures suggestive of testicular cords develop in the medullary parts (63). These results are comparable to those obtained in birds, but are not as clearly understood: germ cells remain oogonia in a chicken ovary cultured with a male gonad (76). In birds, as in amphibia and reptiles, the inversion of ovarian germinal cells with hormones is itself a classic feature. Burns' (21) experiment in the opossum constitutes the only case of production of an ovotestis after estrogen injection. Just as oogonia from the right or left ovary become oocytes *in vitro* (77), the normal right *in vivo* involution would result from an inhibiting action from the left ovary. In the sheep cultured ovary, an unfavorable somatic cell-germ cell ratio could be developed.

5. A nonprimary hypophyseal influence is indicated in that only a slight delay of the meiotic processes occurs in the decapitated or hypophysectomized sheep embryo (105). The addition of gonadotropic hormones (PMSG, crude hypophyseal extracts of embryos and adults of the same species, pure FSH, LH, and prolactin or a combination of the three hormones in culture medium) does not modify the *in vitro* evolution of oogonia in sheep (105, 109) or in hamster (25). However in adult lorises, the total number and the proportion of oogonia compared to oocytes varies with the physiological state (2), and the total number of germ cells increases after estrogen treatment (2). Labeled oocytes are present a short period of time after radiotracer injection in such follicles

but are absent in the untreated animals (33). Both estrogen and FSH might interfere with the development of somatic cells, and, thus, with the production of oocytes.

### III. Folliculogenesis

#### A. RELATIONSHIPS BETWEEN OOCYTE AND FOLLICLE

##### 1. Relationship between the Size of the Oocyte and of the Follicle

The growth of the oocyte can be divided into two phases, relative to that of the follicle (18). The first is phase A in which the oocyte grows to approach its maximum size. Simultaneously, the multiplication of follicular cells occurs. During the second phase B, the oocyte increases very slowly in size, but the follicle grows rapidly in size. The secretion of the follicular cells commences at the beginning of follicular growth, during the first phase (57), and intercellular spaces develop.

These spaces in the ovarian follicle of the normal adult cow and rat increase during follicular growth and a strict linear relation, especially in the cow, exists between the size of the antrum and the size of the whole follicle. However, a great variability of morphogenetic parameters exists between follicles. Some follicles continue to grow without formation of an antrum, while others develop an antrum too early during their growth (96). After hypophysectomy, the oocyte reaches its adult size in follicles persisting for 9 days, although these follicles are smaller than those found in the normal animal. This diminution is the result, first, of the disappearance of secretory function, and second, of a decrease of follicular cell multiplication.

##### 2. Metabolism of the Oocyte during Growth and Maturation

The increase in size of the oocyte corresponds to an intense metabolic activity of the cell, but it exists only during phase A of follicular growth. The level of RNA in the oocyte is low during this whole phase (31, 32, 59). The incorporation of tritiated uridine is highest in the growing oocyte (120). The course of tritiated uridine incorporation follows that generally observed in other cells: successive labeling of the nucleolus, the nucleus, and then the cytoplasm. One hour after injection of the radioactive tracer, oocyte nucleolar and nuclear synthesis increases linearly with oocyte size and reaches a peak in fully grown oocytes at the end of phase A of growth (114). Fluorescence microscopy of the nucleolus indicates a very high level of nucleolar RNA during this phase of oocyte growth; this level

diminishes considerably at the time of oocyte atresia or before maturation as shown by nucleolus vacuolization (91), staining affinity (48), or green fluorescence with acridine orange limited to an envelope.

The existence of active synthesis of proteins does not exclude the possibility of macromolecular transfer during this phase of oocyte growth (51).

Exchanges between oocytes and perioocyte cells have not been clearly defined for the second phase of growth. However, the incorporation of  $^{35}\text{S}$ -methionine (86, 115) and of  $^3\text{H}$ -phenylalanine (150) by rabbit oocytes a little before meiosis is proof that such exchanges do take place. This phase of follicular growth is characterized by the secretion of follicular fluid, particularly of mucopolysaccharides by the granulosa cells, as shown by the sequence of  $^{35}\text{S}$ -sulfate incorporation by the follicles (122). The rapid preovulatory growth is characterized by modification of the permeability of the follicular blood-fluid barrier (172, 173).

Finally, a zymogen enters the extracellular compartment via cytoplasmic processes of fibroblasts. A gross reduction in the tensile strength of the collagenous layer occurs. This collagenolysis ceases after ovulation, perhaps caused by serum antiproteases (44). Deterioration of the follicle wall depends upon enzymes, steroids, cyclic AMP, and prostaglandins, but the precise sequence of reactions is still open to discussion (44, 149).

### 3. Functional Interactions between Oocyte and Granulosa Cells

The follicle constitutes a balanced physiological unit the oocyte preventing luteinization of the granulosa and theca cells (119) and the granulosa inhibiting the resumption of oocyte meiosis (49). This reciprocal control is responsible for the diestrus stage for a long period. Experimental detaching of the oocyte and its culture, or spontaneous detaching by granulosa pyknosis during atresia are followed by meiotic metaphase II. These oocytes can be fertilized, after which they develop abnormally due to an incomplete cytoplasmic maturation. The two steps may be considered in a complete oocyte maturation; the first one, i.e., the resumption of meiosis, is not basically dependent on gonadotropins; the second one, i.e., the passage of the male pronucleus growth factor (MPGF), on the contrary, or its precursor inside the oocyte is gonadotropic follicular dependent (164).

## B. DEVELOPMENT OF DIFFERENT FOLLICULAR TYPES

### 1. Definition of Follicular Types

a. MORPHOLOGICAL CRITERIA. Pincus and Enzmann (140), have classified follicles according to histological criteria: thickness of the theca and



granulosa, organization of the latter around the oocyte, degree of vascularity, quantity of muscle fibers and interstitial tissue, and position of the antral cavities. The classification most widely used until 10 years ago was that of Mandl and Zuckermann (93): follicles with one layer of flattened cells, one layer of cuboid cells, two, three, or four layers of cells, and follicles with or without antra.

**b. DIMENSIONAL CRITERIA.** Follicles with an antrum can be differentiated by measuring their diameter or calculating their volume (14, 36, 142). The size of the classes account for the exponential growth of a follicle and are equal in a logarithmic scale. Using a similar hypothesis that the number of cells increases as an exponential function of follicular age, Peters and Pedersen (135) consider seven classes delimited by cell numbers. However, the two extreme classes are poorly defined. For the largest ones, Mariana proposes the antral surface to the whole surface ratio (96) or four parameters for a definition of the distributive homogeneity of the granulosa cells in space (94). For the smallest ones, the descriptive morphology of follicular cells or their spatial organization is useful for a definition of early growth as the follicle with a round cellular nucleus incorporates tritiated thymidine. However, the population of primordial follicles, defined by follicular cells with flattened nuclei is heterogeneous: the frequency curve of nuclear size is clearly bimodal, and nuclear growth is indicative of the beginning of oocyte evolution.

**c. NORMAL AND ATRETIC FOLLICLES.** The involution of a follicle can be defined by a series of morphological changes; they appear either in the granulosa somatic cells, or in the oocyte, and lead ultimately to the disappearance of the follicle. The characteristics of the granulosa cells during atresia of follicles with an antrum or with several cell layers are pycnosis, replacement by cells with small fusiform nuclei, and disruption of relationships between granulosa cells, antrum or oocyte.

These histological changes are preceded by histoenzymological changes. In the granulosa, the dissociation of cells bordering the antral cavity is accompanied (i) in the rat by the appearance of acid phosphatase and aminopeptidase activity (87), organophosphate-resistant esterases (19, 20), and enzymes probably connected with lysosomes; (ii) in the guinea pig, nucleoside polyphosphatase activity (both ADPase and ATPase) (1); or (iii) more generally by sudanophil lipids, or lipids stainable with red oils (34, 35, 56, 57). The increase in alkaline phosphatase activity in atretic follicles of the cow ovary (116) has not been confirmed in the rat.

Among such a large number of involutive characteristics, the presence of a few pycnotic cells was previously considered to be the practical criterion.

## 2. Dynamics of Follicular Growth

a. **MODEL OF FOLLICLE KINETICS.** The evolution of the distribution curve of the follicular size classes as a function of time depends upon the follicular growth rate, the number of follicles starting to grow, and atresia.

Such a mechanism can be represented by a simple model in discrete time (97). If  $a(o, t)$  is the number of follicles which begin their growth at time  $t$ , the expected value of  $n_{i,t}$  numbers of follicles at stage  $i$  and time  $t$  is

$$E(n_{i,t}) = a(o, t - i)P_{(t-i, i-1)}$$

where  $P_{(t,i)}$  is the survival probability for a follicle at stage  $i$  at time  $t$ , with

$P_{(t,i)} = \prod_{j=1}^i p_{(t-j, t-j)}$ . An estimation of the difference  $p_{(t,i)}$  is given by the proportion  $\hat{p}_{(t,i)}$  of the normal follicles at a stage  $i$  and time  $t$ . If  $n_{i,t}$  is the total number of follicles,  $n'_{(i,t)}$  the number of follicles in early atresia,  $\hat{p}_{1,t} = n_{1,t}/n_{i,t} + n'_{1,t}$ .

Such a model assumes that the beginning of follicle growth is a continuum (139). The proposal that follicles grow to a certain stage of development but are arrested to form a pool of large reserve follicles from which the preovulatory follicles are recruited (55) is unacceptable. Unlabeled large follicles in immediate autoradiographies or heavily labeled large follicles in delayed autoradiographies have never been found after tritiated thymidine injection during the immature as well mature period in mice (23, 129, 134) and in mature rats.

The use of a dimensional criterion gives a frequency curve with a constant shape which can be explained by the previous parameters of follicle kinetics, but mainly by the great variation in follicle atresia (62).

b. **DURATION OF DEVELOPMENT AND REGRESSION OF FOLLICULAR TYPES.** Using flash labeling with tritiated thymidine at the time of estrus, Peters and Levy (134) showed that in the mouse the follicles starting to form a large antrum are those that ovulate in the succeeding cycle; those not yet having an antrum, or having one that is still growing, ovulate during the four subsequent cycles (Fig. 3).

Growth rates can be calculated by the doubling time of granulosa cells determined from their labeling index and the duration of their DNA synthesis phase. A follicular developmental curve takes 14 days to grow from a stage 3h to a stage 5a of Peters and Pedersen's classification. The preovulatory stage is reached in 5 more days (127). Moreover, the follicles grow faster early in life (14 days postnatal) than later (28-35 days postnatal) and faster during estrus than during the metestrus period (128).

In domestic mammals, observations given by the variation in the num-

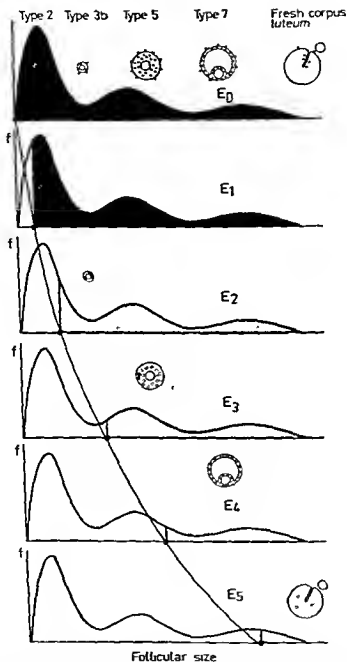


FIG. 3. A schematic representation of the labeling dilution after a flash injection of tritiated thymidine on the day of estrus in mice from Peters and Levy's results (131). The frequency curves are the standard ones and the follicle types refer to Peters and Pedersen's classification. The spots on the follicles have an intensity in relation to the labeling as also expressed by the blackness of the dotted part of the curves. The letter E gives the number of the estrous cycle after the radiotracer injection. The smallest follicles labeled immediately ovulate after five estrous cycles but with a poor labeling intensity.

ber of the large antral follicles during the estrous cycle allowed calculations of the last phase of growth. In the ewe, it is assumed that the ovulatory follicle starts to grow between 4 hours and 5 days after the start of the cycle. It then pauses, and ends up with a phase of rapid growth lasting a few hours at the beginning of estrus (65). More precisely, from the doubling time of granulosa cells estimated by the mitotic index and mitotic time obtained by the use of colchicine, a follicle takes about 5 days to grow from 0.5 to 2.2 mm diameter and an additional 4 days to reach 4.5 mm diameter (K. E. Turnbull and A. W. H. Braden, personal communication). By labeling with india ink the 4.5 mm follicles present in the ovaries at three different times during the estrous cycle, the follicle which finally ovulates grows to the 5-mm size during the 36 to 48 hours before the onset of estrus. The preovulatory enlargement up to 12 mm occurs after the beginning of estrus (160).

Few data exist on the duration of atresia. In mice, the time it takes a follicle without signs of atresia to go through progressive stages of atresia is about 4 days (23).

**c INITIATION OF FOLLICLE GROWTH** In the mouse, around the time of birth, all oocytes are organized and enclosed in small follicles with few follicular cells. Ovigerous nests have disappeared and a pool of non-growing follicles is formed. Ovaries explanted on day 2 postpartum and cultured without the addition of exogenous gonadotropins show groups of oocytes surrounded by a basement membrane and a few small follicular cells. These structures are reminiscent of the polyovular follicles seen in the opossum and hamster ovaries (7). In piglets, treated after birth with progestagen, i.e., with a blocked gonadotropin release, oocyte nests persist longer than in controls (156).

Follicles start to grow at all times and at all ages but more follicles start to develop during the first 3 weeks of life than at later ages (127). Gonadotropin does not influence follicle growth initiation as neither exogenous (137) nor the blocking of the endogenous gonadotropin in androgenized mice alters the number of the first growing follicles (138). The beginning of follicle growth seems to be controlled by an intraovarian regulation in which a substance freed by degenerating large follicles has a reducing effect on growth initiation (138).

The absolute number of "starting" follicles in 7-day-old rats is influenced by the size of nongrowing follicles and by a stimulating factor which differs between strains and is still unknown (95).

**d RELATIONSHIP BETWEEN THE DIFFERENT CATEGORIES OF FOLLICLES** The size of the primordial follicle pool has an influence on the number of "starting" follicles that begin growth in mice (82) and in rats (95).

as does a pool of nongrowing follicles artificially reduced at an early age by radiation or application of dimethyl benzathracene (82) or after testosterone injection on day 5 (136).

In the ovaries of prepubertal mice, the number of growing oocytes decreases in parallel with the number of primordial follicles (69) and represents a constant proportion (11%) of the total number of oocytes (130-133). The same may be true of adults, since after the fourth year in the cow, the growing follicles diminish in the same way as the primordial follicles (38, 43). Similarly, in the rat, the number of follicles larger than 20  $\mu\text{m}$  is related to the number of smaller follicles (3), and in women up to 35 years old this relationship exists between follicles smaller than 100  $\mu\text{m}$  and primordial follicles (15).

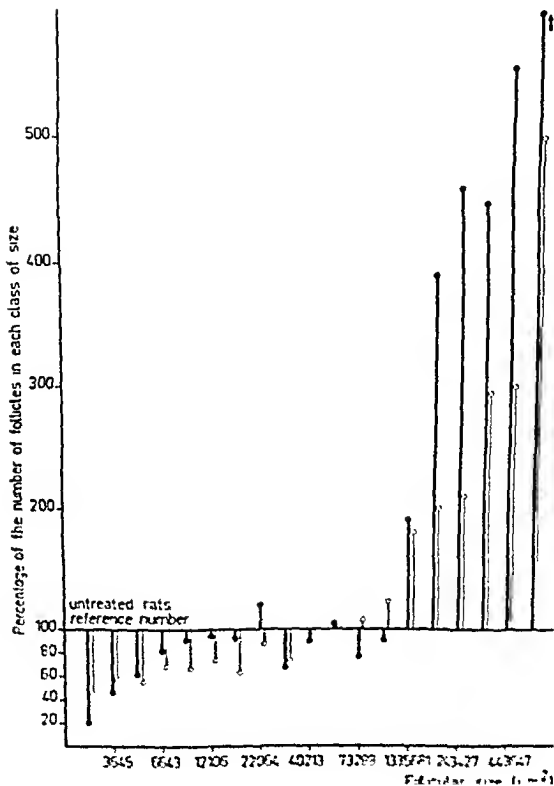
However, the number of primordial follicles is not as dependent on the number of normal antral follicles due to the extent of atresia. During the immature period, many follicles start to grow, and at 3 weeks in mice, 24 days in rats, 3 months in calves, and 1 month in lambs, the ovary contains an unusually large number of large follicles (127, 128, 135). Most of them become atretic. In the adult animal, the growth of the "privileged" follicle is always accompanied by the disappearance of the other follicles in the cow, sow (103), hamster (53, 54), guinea pig (1, 46, 118), and in the ewe (65). In the cow, large numbers of atretic follicles with antra are observed at the 10-12th and 12-14th day of the estrous cycle (137) just as it is surmised that FSH is responsible for the recruitment of a set of follicles from the pool of growing follicles, the hormonal conditions which lead to ovulation must change the relationship between follicles of different sizes.

In fact, breed differences or genetic selection for the incidence of multiple births in sheep or hormonal conditions which lead to spontaneous or induced ovulation of large numbers of eggs in rats, also lead to relatively small populations of oocytes at birth (83, 84), at 7 days, or 5 months of age (166). Paradoxically, the strain of rat with the least follicular development during the prepubertal period has the highest concentrations of FSH and estrogen in blood plasma (147).

### C. DEPENDENCE OF THE FOLLICULAR GROWTH ON HORMONAL FACTORS

By definition, follicular growth is dependent on FSH (45). Growth is stimulated by FSH or PMSG in follicles of all sizes as revealed *in vitro* studies of mouse ovaries, by treatment of immature hypophysectomized rats (145, 146) or of intact mature rats (Fig. 4).

In the rat as well as the cow, PMSG rapidly changes growing follicles into antral follicles and modifies the percentage of volume occupied by the



antrum in a follicle of a precise size (96). Similarly, in immature hypophysectomized rats, FSH acts on the intercellular spaces, causing a displacement in the modal peak of the frequency curve of follicles (Fig. 4). FSH and PMSG have a specific action on antral formation (145). These two hormones also increase cellular multiplication as shown by the augmentation of the labeling index of the granulosa cells (146) and by a correlative increase of the number of cells in the largest follicles from the PMSG-treated rats (96). The ability of large follicles to ovulate seems to be due to an adequate relationship between the volume of antrum and the cellular mass. FSH and PMSG could prevent, as well as stimulate, atresia. LH and HCG allow a more potent action of FSH on follicular growth, increasing the FSH receptors to estrogens in the follicle (52).

The follicle determines its development and its fate (155) from the steroid concentration in its antral fluid.

In conclusion, female fertility does not depend directly on the ovarian follicular population except in extreme cases. During the period of establishment of the ovarian structure, differences in follicular population can interact with the maturation of the hypothalamic-hypophyseal complex and lead to definitive adult possibilities in ovulation rate. The recent demonstrations that exchanges exist between somatic and germ cells have provided a new and important insight into the organization of follicular structure and intraovarian regulation.

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# 8

## Spermatogenesis in Domestic Mammals

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### I. Introduction

Spermatozoa represent only the final step in a series of complex changes (spermatogenesis, 29) that govern their number and properties. We will try to clarify the phenomenon of spermatogenesis in the bull (54), the ram (81), the boar (96), the horse (98), and the rabbit (4, 97).

1 The primordial germ cells migrate from germinal crests and come to lie in the gonads sometime before sexual differentiation. In the fetus and young male the gonocytes are contained inside the seminiferous tubules.

2 In the species being considered, gonocytes multiply and, some months

after birth, give rise to *spermatogonia*. The quantitative efficiency of spermatogenesis depends to a great extent on the manner in which these divisions take place.

3. The cells originating from the last spermatogonial division are the *primary spermatocytes*. Meiotic division of these spermatocytes results in the production of daughter cells, the *secondary spermatocytes*.

4. The metamorphosis of the *spermatids* (products of the division of the secondary spermatocytes) into spermatozoa (spermiogenesis) constitutes the fourth point of interest. The quality of the spermatozoa produced depends to a great extent on this metamorphosis, which occurs in the seminiferous epithelium, but undergoes completion in the epididymis.

5. The various germ cells are located in the seminiferous epithelium, whose structure is maintained by the Sertoli cells.

## II. Description of the Spermatogenic and Seminiferous Epithelial Cycles

The spermatogenic cycle begins with a stem cell or A-type spermatogonium, which provides the starting point of a spermatogenic series. Before this series has completed its evolution, several new ones start in the same part of the seminiferous tubule. Thus, any section of a seminiferous tubule shows several superimposed generations of germ cells. They develop in close relation to one another such that in any given area of the seminiferous epithelium there is a constant succession of cellular associations that takes place with a cyclic regularity.

The cycle of the seminiferous epithelium is formed by the series of changes occurring in a given area of the seminiferous epithelium between two successive appearances of the same cellular association. The duration of the spermatogenic cycle is the interval between the appearance of the stem spermatogonium and the release of spermatozoa which are produced from it. It thus represents the length of time necessary for the formation of the spermatogenic series.

### A. THE STAGES OF THE CYCLE OF THE SEMINIFEROUS EPITHELIUM

The cellular associations which may be recognized during a cycle of the seminiferous epithelium permit the various stages to be distinguished. Two principal methods of classification may be used. One is based on the development of the acrosomic system (22, 63), while the other is based on the

morphological changes of germ cell nuclei and local arrangement of spermatids (81, 92).

## 1. Classification Based on Acrosome Development

With this system, the number of stages varies between or within species (23, 11). Clermont and Leblond (23) defined twelve stages in the cycle of the seminiferous epithelium of the ram and of the bull. In the uniformly stained idiosome (stage 1) of the young spermatids, two or three pro-acrosomic granules appear (stage 2) which fuse into one single acrosomic granule (stage 3) during the "Golgi phase." The "cap phase" is characterized by a slight flattening of the granule on the nuclear surface (stage 4), then by the appearance of a head cap (stage 5), which gradually covers, first, one-third (stage 6), then one-half (stage 7) of the nuclear surface. At the beginning of the "acrosome phase," the acrosomic granule and the cap migrate toward the basement membrane (stage 8). Following this, the acrosomic granule, which is now known as the acrosome, protrudes at the tip of the nucleus (stage 9) and changes from an elongated rod (stage 10) into a triangle (stage 11) and, finally, into a crescent (stage 12). The "maturation phase" therefore, succeeds the "acrosome phase."

## 2. Classification Based on Germ Cell Association

According to Roosen-Runge and Giesel (92) and Ortavant (81), eight stages may be defined in the seminiferous epithelial cycle of the ram (Fig. 1), bull, and boar, which are described below.

Stage 1. From the end of the spermatozoa release into the lumen until the beginning of the spermatid nuclei elongation. It is characterized by the presence of spermatids with round nuclei only (Fig. 2).

Stage 2. From the elongation of the spermatid nuclei up to the formation of the bundles of spermatids. This is the phase of nuclear elongation of the spermatids (Fig. 3).

Stage 3. From the formation of the first elongated spermatid bundles in the Sertollian cytoplasm up to the first maturation divisions (Fig. 4).

Stage 4. From the appearance of the first divisions to the disappearance of the second maturation divisions (Fig. 5).

Stage 5. From the end of the last maturation divisions up to the appearance of dusty chromatin in the nuclei of the young spermatids; during this stage the latter have a small nucleus containing some karyosomes connected by a chromatin network.

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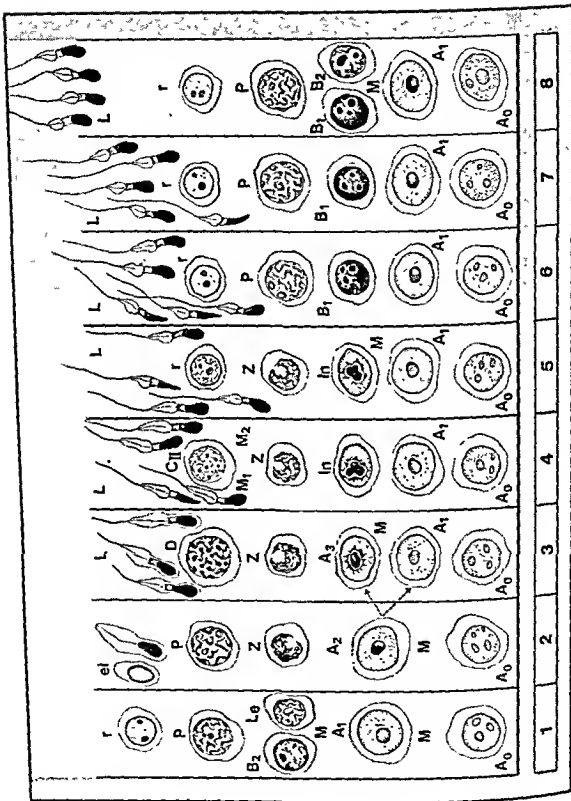
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Stage 4 From the appearance of the first divisions to the disappearance of the second maturation divisions (Fig 5)

Stage 5 From the end of the last maturation divisions up to the appearance of dusty chromatin in the nuclei of the young spermatids, during this stage the latter have a small nucleus containing some karyosomes connected by a chromatin network.





Stage 6 From the appearance of the dusty chromatin in the young spermatids up to the migration of the bundles of elongated spermatids toward the lumen of the seminiferous tubules

Stage 7 From the beginning to the end of the centripetal migration of the elongated spermatids toward the lumen (Fig 6)

Stage 8 From the end of the migration of the spermatids to their complete release as spermatozoa into the lumen (Fig 7)

Stages 1 to 3 are characterized by the presence of a single generation of spermatids, and stages 5 to 8 by two generations

For precise analysis, the longest stages in a given species may be subdivided [stage 5 in the rat (33), stages 1, 2, 3, 4, and 8 in the bull (53)] This last method permits a rapid identification of variations in the principal events of spermatogenesis

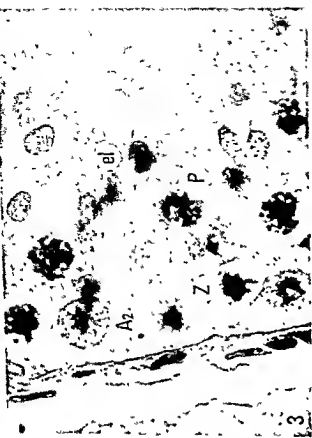
## B FREQUENCY OF THE STAGES OF THE SEMINIFEROUS EPITHELIAL CYCLE

Seminiferous epithelial cycles are similar in the bull, the ram, and the rabbit, they differ in the boar and horse and tend to resemble the pattern of the cycle in the rat (Table 1) The rate of occurrence of the first three

TABLE 1  
Frequency of the Stages of the Seminiferous Epithelial Cycles

Stage	Bull (52)	Ram (87)	Boar (96)	Horse (98)
1	30.8 ± 0.3	21.7 ± 0.9	10.8 ± 0.3	16.9 ± 0.4
2	9.1 ± 0.2	10.6 ± 0.6	14.4 ± 0.3	14.9 ± 0.4
3	20.1 ± 0.2	18.4 ± 0.6	3.5 ± 0.2	3.2 ± 0.2
4	12.8 ± 0.2	10.5 ± 0.5	11.6 ± 0.3	15.8 ± 0.4
5	1.6 ± 0.1	4.2 ± 0.4	8.9 ± 0.3	7.4 ± 0.4
6	5.9 ± 0.1	13.1 ± 0.8	20.3 ± 0.4	13.5 ± 0.5
7	8.1 ± 0.1	10.8 ± 0.7	18.5 ± 0.4	12.6 ± 0.6
8	11.6 ± 0.2	10.3 ± 0.6	12.0 ± 0.3	15.7 ± 0.5

FIG 1 Cellular composition of the stages of the seminiferous epithelial cycle in the bull Each column (1 to 8) shows the germinal types present in a given cellular association These associations or stages are identified by the morphological changes of germ cell nuclei and local arrangements of spermatids A<sub>1</sub> and A<sub>2</sub> reserve and renewing stem cells respectively A<sub>1</sub> and A<sub>2</sub> type A spermatogonia In intermediate type spermatogonia B<sub>1</sub> and B<sub>2</sub> type B spermatogonia M close to a spermatogonium means a mitosis Le Z P and D, leptotene zygotene pachytene and diplotene primary spermatocytes respectively C<sub>1</sub> secondary spermatocytes M<sub>1</sub> and M<sub>2</sub> first and second meiotic divisions r, el L, round elongating and elongated spermatids respectively



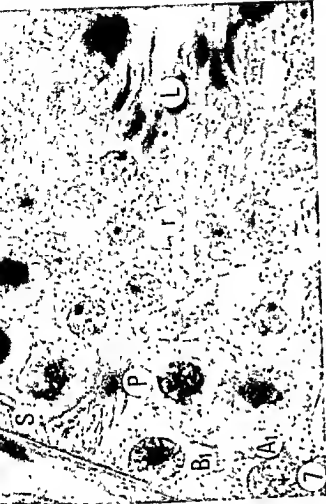
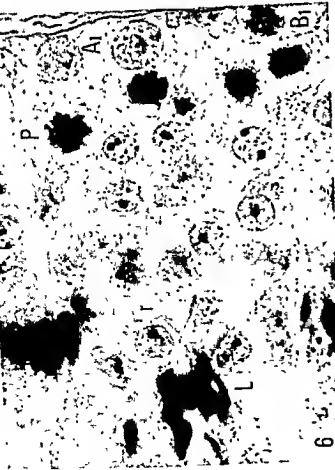


FIG. 2. Stage 1. Spermatid nuclei are round (r); two generations of primary spermatocytes are present: pachytene (P) and pachytene (P). A<sub>1</sub>: spermatogonium; B<sub>1</sub>: basal membrane.

FIG. 3. Stage 2. Spermatid nuclei are beginning to elongate (el). Zygote (Z) and pachytene (P) primary spermatocytes. A<sub>2</sub>: spermatogonia.

FIG. 4. Stage 3. Bundle formation of elongated spermatid (L) toward Sertoli cell nuclei (S). Zygote (Z) and diplotene (D) primary spermatocytes. A<sub>3</sub>: spermatogonia; S: Sertoli cell nucleus.

FIG. 5. Stage 4. Bundles of elongated spermatids (L). C<sub>1</sub>: secondary spermatocytes; M<sub>1</sub>: first meiotic divisions; Z: zygote primary spermatocytes.

FIG. 6. Stage 7. Two generations of spermatids. Bundles of elongated spermatids (L) are moving toward the lumen of the seminiferous tubule; spermatid with round nuclei (r). One generation of primary spermatocytes at pachytene stage (P). A<sub>1</sub> and B<sub>1</sub>: A<sub>1</sub> and B<sub>1</sub> spermatogonia.

FIG. 7. Stage 8. The heads of immature spermatozoa (L) line up the inner surface of the seminiferous epithelium: spermatid with round nuclei (r). P: pachytene primary spermatocytes; B<sub>1</sub>: spermatogonium; A<sub>1</sub>: spermatogonium; S: Sertoli cell nucleus. When the spermatozoa have been released, the seminiferous epithelium is again at stage 1 of the cycle.

stages decreases from the bull to the horse (62.2–35.0%), while that of the last four stages increases (26.2–49.2%). It therefore appears that the relative frequency of the different parts of spermiogenesis varies among species. However, the rate of occurrence of the various stages in the seminiferous epithelial cycle is constant for one species (52).

### C. THE SPERMATOGENIC WAVE

The succession of cellular associations takes place not only in a cross section but along the length of the seminiferous tubule as well (51, 64, 85). Therefore, a portion of tubule displaying one type of cellular association is followed by a portion of tubule displaying the stage immediately preceding or following it in the seminiferous epithelial cycle. There is a "continuity of the segmental order" (85). Each complete spatial series of cellular associations is called a "spermatogenic wave." In some places the spermatogenic wave displays local reversal in the order of the consecutive stages. These irregularities are called "modulations" (51, 85).

The tubular length occupied by one stage of the seminiferous epithelial cycle is not constant. As a result a variation in the length of the spermatogenic wave exists. Its average length is 10 mm in the bull. Nevertheless, it has been observed that for each stage the relative ratio of its mean segmental length corresponds to its relative frequency determined on cross-sectioned tubules, i.e., to their duration (51, 85). A well-defined wave does not exist in man (19, 105).

## III. The Cellular Elements of the Spermatogenic Cycle in Domestic Animals

After the divisions of germ cells, cytokinesis is incomplete so the daughter cells remain connected to each other by cytoplasmic bridges.

### A. THE SPERMATOGONIA

Spermatogonia can be defined as the germ cells which arise from the gonocytes and are contained in the parietal layer of the seminiferous tubules. Their last generation gives rise to the primary spermatocytes.

#### 1. Morphology of the Different Classes of Spermatogonia

Several types of spermatogonia may be distinguished: the dustlike (90) or type-A spermatogonia (3), the intermediate-type spermatogonia (22), and the crustlike (90) or type-B spermatogonia (3). The type-A sper-

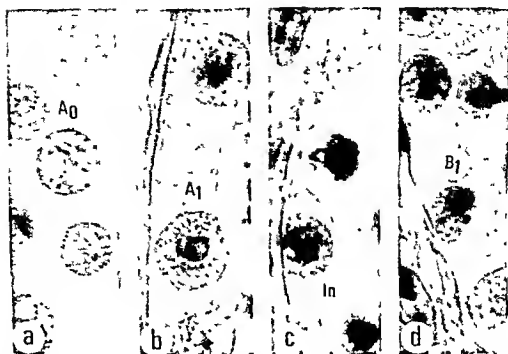


FIG. 8. Bull spermatogonia (Alcian blue, Feulgen,  $\times 850$ ) a: Group of  $A_0$  spermatogonia with pale and round nuclei b:  $A_1$  Spermatogonia with pale and ovoid nuclei containing one central nucleolus. c: Intermediate spermatogonium (In) with more stainable chromatin round the nucleolus d:  $B_1$  Spermatogonia with round nuclei containing crust of stainable chromatin in the nucleoplasm along the nuclear membrane and around the nucleoli.

matogonia  $A_0$  to  $A_3$  (Fig. 8a and b) are large cells, more or less flattened against the wall of the seminiferous tubule. The nuclei, each surrounded by a thin envelope, are ellipsoid in shape with very fine chromatin granules and a large nucleolus. The nucleus of the intermediate-type spermatogonia contains a coarser chromatin (Fig. 8c). The type-B spermatogonial nucleus (Fig. 8d) is smaller and increasingly spherical. The chromatin granules, which are abundant in the ram, but scarcer in the bull and the boar, tend to adhere to the nuclear envelope.

The duration of DNA synthesis increases progressively from type-A to intermediate-type and type-B spermatogonia (50, 53, 70), being, respectively, 10, 16, and 20 hours for the three categories of spermatogonia in the bull (53).

The chromosomes differ in appearance in each spermatogonial division. They are elongated during the prophase of the type-A spermatogonia and are shorter and contracted during the prophase of the type-B spermatogonia.

## 2. Spermatogonial Renewal

During each cycle, new spermatogonia must appear to replace those which develop into spermatogenic series. The problem is to define the

TABLE II

Variation of the Mean Corrected Number of Spermatogonia per Cross Section (10  $\mu$ m) of Seminiferous Tubule in the Bull

Stages	Spermatogonia	Mean number $\pm$ S.E.M.
1-8	A <sub>0</sub>	0.35 $\pm$ 0.12
5-1	A <sub>1</sub>	2.22 $\pm$ 0.15
2	A <sub>2</sub>	4.60 $\pm$ 0.10
3	A <sub>2</sub>	5.63 $\pm$ 0.29*
4	tn	8.37 $\pm$ 0.28
7	B <sub>1</sub>	13.78 $\pm$ 0.99
8	B <sub>2</sub>	20.62 $\pm$ 0.67

\* The division of A<sub>2</sub> spermatogonia gives rise either to new A<sub>1</sub> or to A<sub>2</sub> spermatogonia. For this reason the ratio of A<sub>2</sub> to A<sub>2</sub> is lower than for mitosis of other spermatogonia.

origin of these new spermatogonia. Type-A spermatogonia at stages 6, 7, and 8 can be considered stem cells. These divide at stages 1-3, and the new stem cells arise from these divisions. In the bull, they originate mostly after the second spermatogonial division by an equivalent mitosis (54) (Table II). In the ram the same pattern is suspected (57). Type-A<sub>0</sub> spermatogonia (Fig. 8a) also divide randomly and can give rise to new type A<sub>1</sub> spermatogonia. This could represent the mechanism by which the stem cell stock increases long after puberty (8). Type A<sub>0</sub> and A<sub>1</sub> spermatogonia are the reserve and renewing stem cells (20).

### 3. Number of Spermatogonial Divisions

In the bull the study of the mitotic index and the frequency of cells labeled with tritiated thymidine show six peaks of DNA synthesis and mitosis: three are type-A spermatogonia, one is intermediate-type spermatogonia, and two are type-B spermatogonia (Fig. 9). A few type-A<sub>0</sub> spermatogonia are also labeled and divide at various stages of the cycle of the seminiferous epithelium. A similar situation exists in the ram (57).

The model of spermatogonial division governs the quantitative production of primary spermatocytes (Fig. 10). The mean observed coefficient of efficiency of spermatogonial mitoses (leptotene/spermatogonia A<sub>0</sub> + A<sub>1</sub>) is 16 for the ram and bull (82), 28 for the rat (18) and for the boar (82), but only 4 for the human male (19).

It is interesting to note that this efficiency coefficient in a bull with a

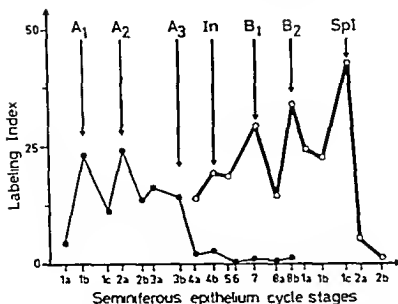
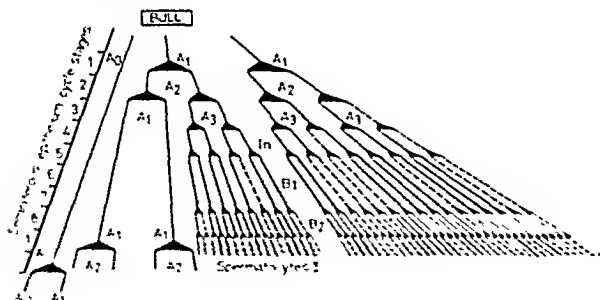


FIG. 9. Variation of the labeling index of the germ cells during the seminiferous epithelium cycle 1 hour after  $^3\text{H}$ -thymidine injection in the bull. Six peaks of labeling correspond to spermatogonia  $A_1$  to  $B_2$ . Few labeled  $A_3$  spermatogonia are observed during stages 4 to 8. Preleptotene and leptotene spermatocytes are labeled during stage 1 (from 53). Spl: spermatocytes I.

subnormal spermatogenesis can decrease to 10. In rams submitted to long daylight, it is reduced to 10 or even less (80). In the ram, the main critical stage is the intermediate-type spermatogonia.





## B. THE SPERMATOCYTES

The primary spermatocytes, products of mitosis of the last generation of spermatogonia, represent germ cells which undergo meiotic division. They appear in mammals either slightly before the release of the spermatozoa into the lumen of the seminiferous tubules [hoar (96), horse (98)] or after [ram (81), bull (53)], but always before the elongation of the spermatid nuclei. Immediately following their formation, the nuclei of the primary spermatocytes show such a great resemblance to those of the mother cells that they have often been mistaken for type-B spermatogonia (Figs. 2 and 6). Some authors have called these cells "resting spermatocytes," but this is incorrect since an active synthesis of DNA [ $S \approx 28$  hours in the bull (53)] and some other compounds (65, 66, 71) occur during this phase. The term "preleptotene" is preferable for this phase.

The chromatin crusts distributed under the nuclear membrane of the primary spermatocytes become dispersed in the nucleus and give rise to thin chromatin filaments "leptotene phase." At the end of this phase in the bull these filaments contract strongly, and the chromosome spirals are reduced to only a few coils (53). Thereafter, the tension of the chromosome spiral diminishes, and long, slender chromosomes are obtained, as in other domestic animals.

At the "zygotene phase," homologous chromosomes pair off and synaptonemal complexes appear, with the exception of the sex chromosomes which are included in the sex vesicle. Chromosomes gather in a bouquet-like arrangement and become even more apparent [stages 3, 4, and 5 of the seminiferous epithelium cycle in the ram and bull (Figs. 3 and 4)].

At the "pachytene phase" each chromosome divides longitudinally into 2 chromatids, thus the chromosomes appear thicker. This phase may be observed at stages 6, 7 (Fig. 6), 8 (Fig. 7), and 1, 2 (Fig. 2) in the ram and bull.

The "diplotene phase" is characterized by the formation of chiasmata between the homologous chromosomes so that they are less easily distinguishable one from another (stage 3, Fig. 4).

In "diakinesis," the final and very short stage of the meiotic prophase, the contraction of the chromosomes is greatest, and each bivalent shows various arrangements.

The end of the meiotic prophase coincides with stage 4 of the seminiferous epithelium, during which the metaphase, anaphase, and telophase rapidly occur. Secondary spermatocytes are now present. They have a spherical nucleus containing five to six particles of Feulgen DNA joined together by a network of filaments. This interphase lasts only a few hours, each secondary spermatocyte then dividing to give rise to two spermatids.

The rate of RNA synthesis is low during the leptotene, zygotene, and early pachytene stages, increasing rapidly during mid-pachytene and the end of meiotic prophase (65).

Meiosis also plays a part in governing the quantitative and qualitative efficiency of spermatogenesis. A certain number of primary spermatocytes do not pass through the zygotene stage; for example, when submitted to long daylight, rams give rise to pycnotic nuclei (80). Another abnormality stems from the absence of the second meiotic division resulting in diploid gametes (9, 38, 46).

The genetic consequences, resulting from the particular behavior of the chromosomes during meiosis, will not be considered here. However, an examination of the behavior of the X-Y bivalent may be interesting. In the domestic animals, the X-Y bivalent is represented by a long X chromosome and a very short Y chromosome. In any case, two categories of spermatozoa are produced. Many authors have tried to take advantage, with conflicting results, of the properties of these two categories of spermatozoa with the aim of controlling the sex ratio (27).

### C. THE SPERMATIDS

Spermiogenesis is the sum of the nuclear and cytoplasmic changes in the spermatids. These changes govern, to a great extent, the quality of the final product: the spermatozoa.

#### 1. Nuclear Changes

According to the morphology, spermiogenesis can be divided into three main periods: round spermatids (stages 5-1), nuclear elongation (stages 2-4), and elongated spermatids (stages 5-8). During the elongating phase the base of the spermatid nucleus of the bull and the ram contracts; no doubt the primary morphological abnormalities of spermatozoa described in the literature are formed at this stage. They can be related to subfertility in the bull (72). The nuclear envelope of the spermatid is double, and contains few if any communicating pores between the karyoplasm and the cytoplasm, even though pores of this type are frequent in the spermatogonia (16). The nucleus of the young spermatid although smaller, is similar to that of the secondary spermatocytes (Figs. 5 and 6). After Feulgen staining, several karyosomes dispersed in a fine dusty chromatin are seen. These granules which, after a while disintegrate into dustlike granulations, become very homogeneous during stage 1 of the seminiferous epithelial cycle in the bull. Then there is some accumulation of the DNA-Feulgen material at the base of the head after its narrowing.

At the beginning of spermiogenesis, the nucleus of the spermatid contains one or more nucleoli. In the ram the rate of incorporation of tritiated uridine is as high in the nuclei of the young spermatids as in those of the diplotene spermatocytes (65). This rate of incorporation decreases gradually, and stops at beginning of the phase of nuclear elongation. Moreover, the RNA synthesized cannot be detected by autoradiography during the succeeding stages (65, 71). During the elongating phase and immediately after it, there is a replacement of the nuclear histones of the somatic-type, rich in lysine, by a new type of histone, rich in arginine (66, 71). Thus incorporation of tritiated arginine and  $^{35}\text{S}$ -cystine has been reported in the ram at stages 4 to 7 of the seminiferous epithelial cycle (67). These changes in the DNA and proteins are accompanied at the end of spermiogenesis by resistance to deoxyribonuclease when an increase in the number of disulfide bonds in the nuclear proteins is observed (67). These rearrangements of the DNA molecules give a crystalline structure to the spermatozoa (109) as they become birefringent (88) and diffract X rays (109).

## 2. The Cytoplasmic Components

The cytoplasm is moderately dense and finely granular in appearance; the endoplasmic reticulum is abundant with smooth vesicles or canaliculi. The ribosomes are scattered individually or in clusters throughout the cytoplasm of the spermatid. As in primary spermatocytes, the mitochondria exhibit unusual structure: their cristae are swollen, occupying most of the organelles as a pseudomatrix (7). When the elongation of the spermatid occurs there is a shift of most of the cytoplasm toward the caudal pole of the nucleus. Finally a certain portion of cytoplasm, containing mostly lipids and RNA particles, becomes separated from the rest of the spermatid. Upon sperm release part of this material forms the residual bodies.

**A. GOLGI APPARATUS AND ACROSOME.** The formation of the acrosomic system is similar in various domestic mammals. Several stages can be distinguished which were used as a basis for a precise classification of the seminiferous epithelial cycle (63).

*i. Golgi phase.* In the young spermatid, the Golgi complex is composed of numerous small PAS-positive vacuoles surrounded by flattened vesicles with parallel limiting membranes (14, 17, 87). Inside this complex two or three proacrosomic granules appear. Both the granules and vacuoles are PAS-positive. The proacrosomic granules gather to form a single *acrosomic granule*, contained in a vesicle (stage 6 of the cycle). They move

toward the anterior part of the nucleus, and the inner membrane of the vesicle adheres to the nuclear membrane, which appears thicker (Fig 11)

*ii Cap phase* Shortly after this, the acrosomic vesicle, flattened onto the nucleus, encompasses the acrosomic granule between its outer and inner membrane. It grows and finally covers nearly two thirds of the nucleus in the ram and boar and a little less in the bull. The remnant of the Golgi complex leaves the acrosomic vesicle and migrates into the caudal part of the cell, often disintegrating during this process.

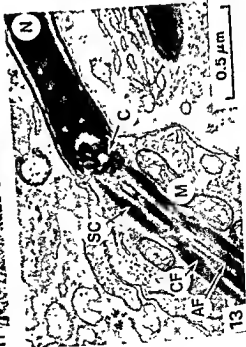
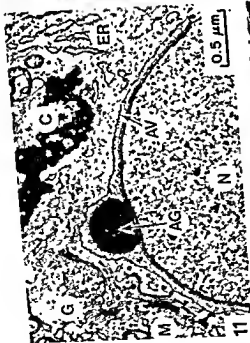
*iii Acrosome phase* Meanwhile, the acrosomic granule flattens gradually onto the nucleus giving rise to the *acrosome* (Fig 12). The combination of the acrosome and the acrosomic vesicle forms the acrosomic system (63), generally referred to as the acrosome.

*iv Maturation phase* Following the elongation of the spermatid, the acrosome undergoes no further modifications except in rodents. By this time the nucleus of the spermatid is surrounded successively by the nuclear membrane, the internal acrosomic membrane, the acrosome body, the external acrosomic membrane, and the cytoplasmic membrane. It is worth mentioning that an anomaly in the acrosomic system causes sterility in the bull (12).

**B THE CAUDAL SHEATH OR MANCHETTE** During stage 2, a transient structure, the caudal sheath, appears in the cytoplasm of the spermatid. It consists of microtubules parallel to the major axis of the cell around the posterior part of the nucleus and the upper part of the flagellum. It starts from the perinuclear ring, a cytoplasmic structure located near the caudal end of the acrosome. The manchette, or caudal sheath, disappears at the end of the elongation of the nucleus (28, 69, 84, 89).

**c THE LOCOMOTORY APPARATUS** This consists of the neck, the axial filament or axonema, and the middle piece. The axial filament is formed from the distal centriole while the acrosomic vesicles appear (16, 106). At the beginning of the elongation of the nucleus, the locomotory apparatus takes up its definitive position at the caudal pole of the nucleus. The formation of the middle piece can be observed afterward (stages 2-7).

*i The neck* (Fig 13) This consists of the proximal centriole and nine cross-striated fibers, four of them fuse 2 by 2 to form the "implantation plate."



ii *The tail* (Fig 14) A proximal section shows that the central part of the axonema is now constituted by two tubular fibrils surrounded by nine "doublets" Each doublet consists of one dense fibril (the A fiber) and one tubule (the B fiber) (39) Nine coarse fibers run close to the fibrillar doublet Three of them (numbers 1, 5, and 6) appear larger than the others (16) In the distal section the two circles of fibers and fibrils are less distinct their outer coarse fibers become thinner and more closely pressed against the corresponding fibrils The coarse fibers numbers 3 and 8 disappear more proximally than the others (16)

iii *The middle piece* During stages 5 and 7, mitochondria assemble in a spiral round the axial filament to form the mitochondrial sheath of the middle piece, between the proximal centriole and the terminal ring (16)

iv *The man piece* The axial bundle of  $9 + 9 + 2$  fibrils contains two longitudinal ribs situated on the same diameter as the central pair of fibrils and surrounded by a helix of structural proteins (74, 100) This whole structure is surrounded by the cellular membrane (16, 61, 110)

#### IV. The Sertoli Cells

The Sertoli cells are the somatic elements of the seminiferous epithelium It is currently assumed that in the adult they do not divide and their numbers do not vary The nucleus is irregular, having a very deep indentation, and its triangular or elongated shape varies during the cycle of the seminiferous epithelium (37, 63) It contains a large nucleolus which is multivesicular in the ram and the bull (29, 75, 111)

Electron microscope observations show that Sertoli cells do not form a syncytium, but that each of them is limited by a distinct membrane (14,

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##### Figs 11-14 Ultrastructure of spermiogenesis

Fig 11 Round spermatid of the ram N nucleus G Golgi apparatus AG acrosomic granule, AV, acrosomic vesicle M, mitochondrion, LR endoplasmic reticulum, C, chromatoid body

Fig 12 Elongated spermatid of the bull showing outward from the nucleus nuclear membrane, inner acrosomic membrane acrosomic material, outer acrosomic membrane cellular membrane and Sertoli cell membrane

Fig 13 Elongated spermatid of the bull N, nucleus, C, proximal centriole in cross section, M, mitochondrion, CF, coarse fibers, AF, axial filament SC, segmented column of the connecting piece

Fig 14 Cross section of a sperm tail in elongated spermatid of the ram (polar view backward from the head), fibers are numbered according to Fawcett (39) (Courtesy of Dr Loir)

40, 106). However, privileged zones of contact become established between neighboring cells, giving them a high cohesion. Thus, Sertoli cells are concerned with the architecture of the seminiferous epithelium. Long, thin cytoplasmic processes surround all the germ cells in the tubule, except the spermatogonia, which are in close contact with basement membrane (16, 76, 102). These processes undergo cyclic variations during the cycle of the seminiferous epithelium. They are concerned with the release of spermatozoa and the resorption of the residual bodies (15, 42, 103). These events could be related to a coordinating role of the Sertoli cells in spermatogenesis. The relationship between the cytoplasm of the Sertoli cells and the germ cells suggest that the former control the exchanges between the germ cells and their environment and regulate the secretion of tubular fluid in relation to the blood-testis barrier (36).

The cytoplasm of the Sertoli cells contains small aggregates of ribosomes and a smooth endoplasmic reticulum, often organized into a network around the nucleus (40, 60). These cells are rich in PAS-positive material, mostly glycogenic in nature (31, 40, 41), and in lipids, whose quantity varies during the cycle (58, 62). The turnover of these lipids is very rapid. In addition, the Sertoli cells are able to metabolize some steroids (10, 34) and elaborate a specific androgen-binding protein (ABP) which transports androgen to the germ cells within the seminiferous epithelium and to the epididymis (91). On the other hand, the Sertoli cells have been suspected as the source of inhibin (32, 43, 101) (see below).

## V. Establishment of Spermatogenesis in the Young Male

The growth curve of the testis (1, 8, 24, 77, 107) has the same appearance in different domestic mammals (Fig. 15). There is a slow growth rate in the first (2 or 3) months after birth followed by a more rapid one thereafter when spermatogenesis commences. At the end of the latter, the eighth or ninth month after birth in the calf, and the fifth month in the lamb, testicular growth slows down.

During the first period only two groups of cells are present in the sex cords: the *supporting cells*, which have a small, highly stainable nucleus and are located along the basement membrane, and the *gonocytes*, whose large and lightly stained nuclei are found in the central part of the sex cords (Fig. 16). The supporting cells are transformed into Sertoli cells and the gonocytes into spermatogonia. The supporting cells proliferate 4 and 6 months after birth, respectively, in the lamb and in the calf, they then stop dividing and differentiate into Sertoli cells. The number of gonocytes increases progressively until adulthood. In the lamb many of them give

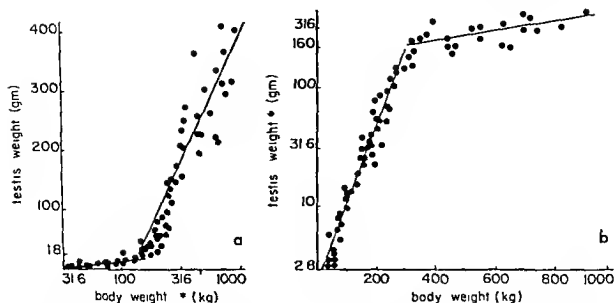


FIG. 15. Evolution of the testis weight in relation to the body weight in the calf.  
 \*: Logarithmic scale (from 8).

rise to type-A spermatogonia, followed, on about the 100th day, by primary spermatocytes, and, about the 120th–125th day, by spermatids. The last stages of the cycle of the seminiferous epithelium occur only toward the 140–150th day (24, 93). In the calf, similar development takes place, but spermatogenesis does not commence until after 4 months following birth; the first spermatozoa appear at about the seventh month (1, 8). Initiation of spermatogenesis is dependent more on the development of the animal than on its age (24, 59, 107). Even at its beginning, spermatogenesis shows similar cyclic changes of the seminiferous epithelium to those

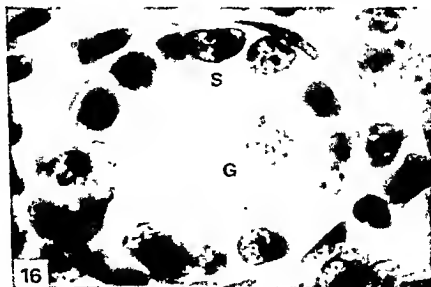


FIG. 16. Sex cord from a young lamb with a gonocyte (G) and supporting cells (S) (from 26).



seen in the adult, but its maximal efficiency is attained only after several months (8, 24).

The establishment of spermatogenesis is, therefore, a very long and progressive phenomenon, in which several stages after the impuberal phase can be distinguished; the whole sequence of events can be summarized by the following (29): (1) impuberal phase, gonocytes; (2) prepuberal phase, cellular differentiation; (3) puberty, release of the first spermatozoa; (4) first postpuberal phase, increase of the spermatogenic yield; (5) second postpuberal phase, adult spermatogenic yield in a still growing testis; and (6) adulthood.

## VI. Duration of Spermatogenesis

The total number of spermatozoa produced per day depends upon the rate of development of spermatogenesis. The duration of spermatogenesis has been measured by following the progression of germ cells labeled with tritiated thymidine through the cycle of the seminiferous epithelium. For example, meiotic prophase takes 15 and 19 days, and spermiogenesis 14 and 20 days in the ram (81) and the bull, respectively (29). Generally, however, results are expressed in terms of the duration of the seminiferous epithelial cycle. This time varies between species (Table III). The rate of spermatogenesis is thought to be nearly constant (21, 33). Thus, it is possible to conclude that when spermatogenesis is disturbed, a certain number of cells degenerate, but those which continue their development do so at the normal rate. The spermatogenic cycle, therefore, seems to be a biological constant. The time taken for the spermatozoa to pass along the ductus epididymis is about 11 to 14 days in the ram (6), 8 to 11 days in the bull (78) and in the horse (98), 9 to 14 days in the boar (94, 96), and 9 to 10 days in the rabbit (5, 79).

TABLE III  
Duration of the Seminiferous  
Epithelial Cycle

Species	Duration (days)
Man (19)	16
Bull (55)	13.5
Stallion (98)	12.2
Ram (81)	10.3
Boar (96)	8.6

## VII. Control of Spermatogenesis

### A. ENDOCRINE FACTORS

The impuberal testis is under pituitary control. The multiplication and differentiation of supporting cells are gonadotropin dependent. In lambs, the testis weight and the number of supporting cells which decrease after hypophysectomy is maintained after injection of LH. FSH has a synergistic action with LH. Testosterone is ineffective (Fig. 17). The mitotic activity of the gonocytes is more pituitary independent but differentiation into spermatogonia, giving rise to spermatogenetic activity with appearance of the primary spermatocytes, is under the control of gonadotropins (25, 26).

In the prepuberal testis of the hypophysectomized rat, spermatogenesis is stimulated by FSH (30) which also induces the secretion of a specific androgen-binding protein (ABP) by the Sertoli cells (44, 48, 108).

In the adult testis of the hypophysectomized rat, LH is able to support spermatogenesis. FSH maintains spermatogonial divisions but not the meiotic prophase or spermiogenesis (30). Testosterone or pregnenolone support complete spermatogenesis (2, 13, 73, 95, 99). This indicates that LH acts through androgenic secretion. Androgen receptors are present in seminiferous tubules (49). Moreover, ABP transports the androgens to the germ cells (86). The seminiferous epithelium secretes inhibin, a protein which regulates FSH. This secretion is negatively related to spermatogenetic activity (32, 43, 101). In conclusion, although the rate of speed

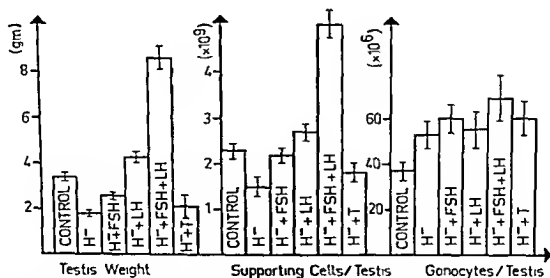


FIG. 17. Variation of the testis weight, the total number of supporting cells, and gonocytes per testis after hormonal supplementation in the hypophysectomized lamb (adapted from 25).

of spermatogenesis cannot be affected by hormones such as gonadotropins or androgens, the number of germ cells produced during spermatogenesis is influenced by these hormones.

## B. EXTERNAL FACTORS

In some domestic species, such as the boar, horse, ram, and rabbit, spermatogenesis shows seasonal variations related chiefly to photoperiod and temperature. In the ram under normal conditions, spermatogenic activity is modulated by daylight duration. It is at a maximum as the daylight decreases from 16 to 10 hours from summer to autumn. It then decreases and remains at a low level until the next summer. Stem and intermediate spermatogonia, pachytene spermatocytes, meiotic divisions, and elongating spermatids are particularly affected (56, 80, 83). It is known that high summer temperatures decrease fertility in cattle, sheep, and swine (35, 47). In the ram and boar when the scrotal temperature is artificially increased to 41°C for 3 hours the pachytene spermatocytes are rapidly destroyed [stage 8 of the seminiferous epithelial cycle (68, 104)]. There is also a transient effect on spermatogonial divisions with an increase of pro-phases and metaphases (104). Some round spermatids also degenerate in the boar (68).

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# 9 Male Reproductive Organs and Semen

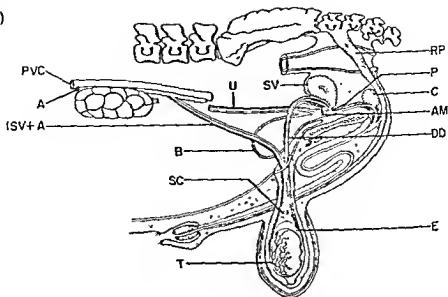
B. P. Setchell

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## I. Introduction

The reproductive organs in male domestic animals comprise the testes, epididymides, and accessory organs (Fig 1) The testes produce the spermatozoa and the male sex hormone testosterone The spermatozoa pass from the testis into the epididymis where they acquire the capacity for fertility and motility and are then stored there At ejaculation, they are squeezed along the ductus deferens into the urethra and are mixed with the secretions of the accessory glands to constitute the ejaculated semen

(A)



(B)

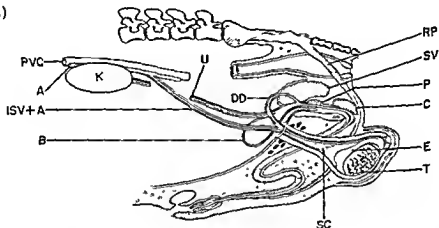


FIG. 1. General view of the reproductive organs of (A) bull and (B) boar. T, testis; E, epididymis; SC, spermatic cord; DD, ductus deferens; ISV + A, internal spermatic vein and artery; A, aorta; PVC, posterior vena cava; AM, ampulla; SV, seminal vesicles; P, prostate; C, Cowper's gland; RP, retractor penis muscle; B, bladder; U, ureter, shown cut, not to obscure the internal spermatic vein and artery, which pass ventromedially; K, kidney. Drawings after Ackerknecht (1).

## II. The Testis

### A. THE SCROTUM AND DESCENT OF THE TESTIS

The testes of the domestic animals originate near the kidneys, but as in many but not all mammals they descend into a scrotum during fetal life. In birds, the testes do not migrate. During fetal life, mammalian testes contain appreciable amounts of testosterone, which is probably responsible for the development of the male or Wolffian ducts. The regression of



the female or Müllerian ducts also depends on the presence of a testis; the active principle is apparently not testosterone, but has not yet been identified (26, 27). It is interesting that in the fetal horse, the testis reaches its maximal size when the fetus is only about 50 cm long (8). The testis of the fetal sheep contains more testosterone and androstenedione at 90 days of fetal life than at birth or before puberty (1a, 2), and the concentration of testosterone in blood serum from fetal calves was higher between 3 and 7 months of fetal life than at 9 months, and higher in male than in female fetuses (6).

The scrotum in adult domestic mammals ranges from a very pendulous scrotum in the ram to a perineal swelling in the pig, but in all these species, it serves to keep the testis several degrees cooler than body temperature. This is achieved by cooling the testicular venous blood during its course near the scrotal skin and then transferring this coolness to the incoming testicular arterial blood in the spermatic cord, which acts as a countercurrent heat exchanger. The scrotal skin is thinner and less well covered with hair or wool than other skin. It can be kept cool by an abundant population of sweat glands and is also well-endowed with temperature receptors which evoke powerful physiological responses in the animal if the scrotum is warmed (49, 50).

If the temperature of the testis is raised to body temperature or slightly above for only a few hours, the animal becomes infertile for some time after a delay of about 14 days. This is because there are cells in the testis which are particularly sensitive to temperature, while the spermatozoa which have left the testis and are stored in the epididymis are less sensitive. The duration and severity of the infertility depends on the temperature and the duration of exposure, and can be permanent. Animals whose testes do not descend into the scrotum, called cryptorchids, or "rigs" are invariably sterile, although they usually retain normal libido because their testosterone production is only slightly subnormal (47, 50).

## B. VASCULAR SUPPLY

The testes of mammals with scrotal testes carry their vascular supply with them when they descend into the scrotum, and in most species, the artery becomes even further elongated by being coiled in the spermatic cord. This coiled artery is surrounded by a multitude of small veins which make up the pampiniform plexus. Once the artery reaches the testis, it does not enter the tissue directly but runs along the posterior border under the epididymis without branching until it reaches the caudal pole of the testis. Then it runs up the anterior border of the testis giving off branches which run into the parenchyma with only minimal branching

until they reach the mediastinum. There they form coils or arches alongside the rete testis and then turn back and begin to branch to supply the parenchyma (40).

The functional significance of this curious anatomy is not fully understood, but several effects have been demonstrated (Fig. 2). The spermatic cord acts as a countercurrent heat exchanger (see above) and eliminates the pulse pressure from the arterial blood with only a small fall in mean blood pressure (40). There is also a suggestion that substances produced in the testis may be recirculated to the testis by crossing from vein to artery in the spermatic cord (19), but this does not appear to be quantitatively important for testosterone in the ram (25) or boar.

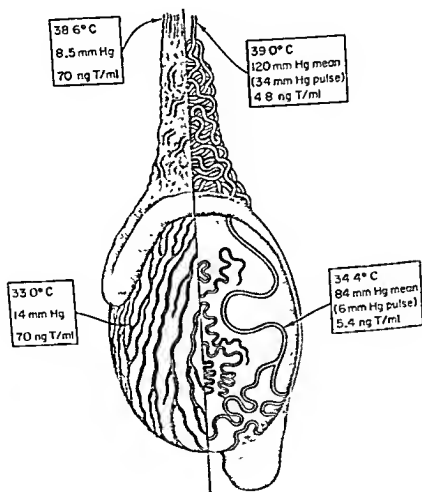


FIG. 2 A diagram of the blood vessels of a ram testis, with arteries on the right side and veins on the left. The figures in the boxes give the temperature, the mean blood pressure (and pulse pressure), and the concentration of testosterone in blood at the point shown. Note the convolution of the artery in the spermatic cord and its sinuous course on the surface of the testis.

Blood flow through the testis is comparatively low so that the testicular venous blood is normally only about 50% saturated with oxygen. Testicular blood flow is unaffected by many factors which cause increases in other tissues but can readily be reduced by catecholamines (40). The blood vessels of the testis are also extremely sensitive to cadmium salts in doses which do not affect other organs; capillary permeability increases and blood flow falls to very low values within hours after a subcutaneous injection (23). Furthermore, capillary or nutrient blood flow in the ram testis is appreciably less than total blood flow (22). The circulation through the testis seems therefore to be rather unusual and warrants additional investigation.

The parenchyma of the testis is encased in a tough capsule, the tunica albuginea, which in many species contains contractile cells and may affect pressure, and therefore fluid movements, within the testis (9).

### C. INTERSTITIAL TISSUE: LYMPH AND TESTOSTERONE PRODUCTION

The parenchyma of the testis is formed mainly by the seminiferous tubules, first properly described by de Graaf in 1668 (10). These tubules are convoluted, roughly cylindrical structures, which are closely stacked together leaving three-sided spaces in between. These interstitial spaces contain the blood and lymph vessels, the nerves, and also the Leydig cells which almost certainly produce the majority of the testosterone. Leydig cells are reasonably sparse in the ram and bull. The lymph vessels in these species are discrete and are usually situated near the centers of the interstitial spaces, quite unlike the large lymphatic sinusoids found in the rodents. In the boar, the Leydig cells are extremely abundant and the lymphatic vessels are quite inconspicuous (18) (Fig. 3). Nevertheless, both the ram and boar testes produce comparatively large amounts of lymph which is very similar to blood plasma in composition, including its high protein content. Moreover, this high concentration of protein is not reduced if lymph flow is increased by venous congestion or locally heating the testis, suggesting that a high protein fluid is filtered by the testicular capillaries. However, their structure is not that of highly permeable vessels and their permeability is not sufficiently high to produce differences in the rate of transit of labeled red cells and proteins; this means that testicular capillaries are not as permeable as liver capillaries (46).

The structure of the Leydig cells is typical of steroid-producing cells. There is an abundant smooth endoplasmic reticulum and prominent mitochondria, fat droplets, and a nucleus. The identification of the Leydig cells as the main source of androgens is based on the production of the hormone *in vitro* by isolated interstitial tissue at a much greater rate than

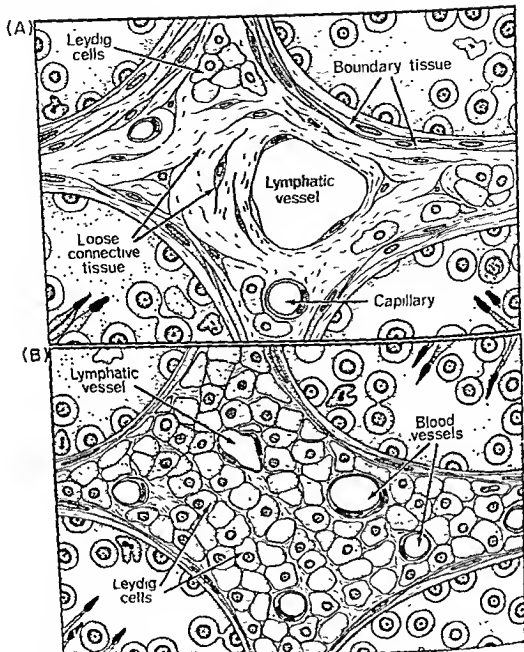


FIG. 3. A diagram of the interstitial tissue of (A) ram and (B) boar testis. In the ram the interstitial tissue contains a central lymph vessel, blood capillaries, and clumps of Leydig cells in a loose connective tissue. Some of the Leydig cells are grouped around capillaries, others are not. In the boar, most of the interstitial space is filled by the numerous Leydig cells with small lymph vessels and blood capillaries in among them. Reproduced by permission of the publishers from Fawcett (14).

by the tubules. Furthermore the steroidogenic enzyme  $3\beta$ -hydroxysteroid dehydrogenase can be predominantly localized in the Leydig cells by histochemical techniques. The testosterone is formed from cholesterol which is either taken up as such from the blood stream or synthesized from

acetate in the Leydig cells. The cholesterol is then converted to pregnenolone in the mitochondria. The remaining steps of testosterone synthesis take place in the smooth endoplasmic reticulum or in the cytosol (7) but a number of possible pathways are available (Fig. 4). Different pathways may predominate in different species or under different conditions.

It is not known how the testosterone from the Leydig cells is transported into the blood, but blood plasma from the internal spermatic vein contains about 20 times as much testosterone as arterial blood does when it enters the testis. There is also a high concentration of testosterone in testicular lymph. It would therefore appear that the tissue fluid bathing the seminiferous tubules contains much more testosterone than does arterial blood. This is an important fact to remember when studying the effects of parenterally administered steroids on the seminiferous tubules. It is necessary to inject much more hormone than the Leydig cells produce to get the same effect in the testis and these large doses then have unphysiological effects elsewhere in the body. However, because of the large differences in flow rate (1200 ml/hour blood, 10 ml/hour lymph in the ram) there is much more testosterone being carried from the testis to the rest of the body in blood than in lymph (40).

The production of testosterone is regulated by luteinizing hormone (LH) from the pituitary (12); human chorionic gonadotropin also increases the concentration of testosterone in both testicular venous plasma and lymph in rams (30). Circulating testosterone, in turn, controls LH secretion by negative feedback (31).

The release of testosterone into the blood is not steady, there are sharp peaks in the testosterone concentration in blood plasma from the internal spermatic vein as well as in peripheral blood. It is not certain whether or not these peaks are associated with fluctuations in release of LH from the pituitary.

In rams there are also important seasonal variations in the rate of testosterone production (28), although this does not vary as widely as in the very seasonal breeders such as red deer (29).

#### D. SEMINIFEROUS TUBULES

The spermatozoa are produced in the seminiferous tubules, which are long convoluted tubular structures, usually two-ended, the ends of which open into the rete testis via the short tubuli recti (Fig. 5). The seminiferous tubules are about 0.20 mm in diameter and in a 250-gm testis of a ram, the tubules total about 7000 m in length, and comprise about 90% of the organ. In the boar, the proportion is much lower because of the greater abundance of Leydig cells. The tubules have a lumen with a

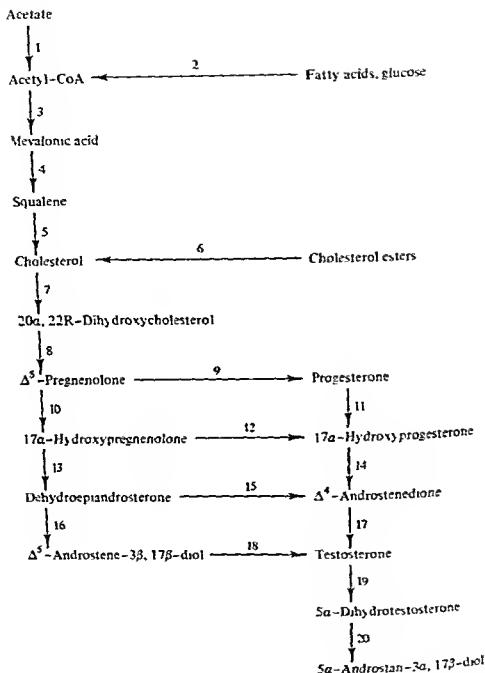


FIG. 4. Pathways of steroid synthesis in the testis. Reactions 6–18 occur mainly in the Leydig cells, reactions 1, 7, and 8 take place in mitochondria; reactions 2, 3, 4, 5, and 9–18 take place in the cytoplasm or in the endoplasmic reticulum; and reaction 6 takes place in lipid droplets in the Leydig cells. Testosterone is then liberated from the Leydig cells into the lymph, blood, and seminiferous tubules. Reactions 19 and 20 occur mostly in the cells inside the seminiferous tubules and the resultant steroids, rather than testosterone itself, may be the active compounds. Reactions 6 and 8 are probably those controlled by LH via cAMP. Reactions 9, 12, 15, and 18 all involve a  $\Delta^5$ - $3\beta$  hydroxysteroid dehydrogenase and a  $\Delta^5$ , $\Delta^4$  isomerase; reactions 10 and 11 involve  $17\alpha$ -hydroxylase; reactions 13 and 14 involve  $C_{17}$ - $C_{13}$  lyase; and reactions 16 and 17 involve  $17\alpha$ -dehydrogenase [see Elk Nes (12)].

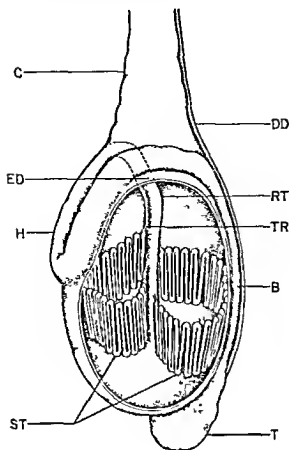


FIG. 5. A diagram of the left testis and epididymis of a ram viewed from the lateral aspect. The arrangement of the seminiferous tubules (ST) inside the testis is assumed to be similar to that in the rat, except that the rete testis (RT) is central in the ram. The seminiferous tubules are joined to the rete by the short tubuli recti (TR). At the dorsal pole of the testis, the rete joins the efferent ducts (ED), which then run into the head (H) of the epididymis, or caput epididymidis. This is doubled back on itself and is closely attached to the surface of the testis. The head leads to the narrow body (B) of the epididymis or corpus epididymidis and then to the bulbous tail (T) of the epididymis or cauda epididymidis. The ductus deferens (DD) arises from the medial face of the tail of the epididymis so its origin cannot be seen on this diagram. It runs up the spermatic cord with the artery and vein, as far as the inguinal canal.

diameter of about 0.08 mm, which is normally filled with fluid. The boundary tissue of the tubules has a clearly defined structure, consisting of an outer cellular layer, an outer noncellular layer, an inner cellular layer consisting of smooth musclelike or myoid cells, and an inner noncellular layer which is particularly well-developed in the ram as a many-layered collagenous matrix (40). Immediately inside the boundary tissue are two types of cells, Sertoli cells and spermatogonia. The latter are confined between the boundary tissue and pairs of Sertoli cells which form specialized tight junctions above them. The Sertoli cells are diploid cells which do not divide in adult animals; the spermatogonia are also diploid, but they divide mitotically to feed cells into the spermatogenic cycle (see Chapter 8). At the beginning of the meiotic prophase, the preleptotene spermatocytes get around or through the Sertoli-Sertoli tight junctions, and spend

the rest of their life sandwiched between adjacent pairs of Sertoli cells with the tight junctions between them and the boundary tissue. During the later steps in spermiogenesis, the late spermatids or immature spermatozoa are embedded in the luminal edge of the Sertoli cell cytoplasm (15, 16) (Fig. 6).

The Sertoli cells fulfill a number of important functions. First, the tight junctions between adjacent pairs of them are probably the major part of the blood-testis barrier (see next section). They are also involved, probably actively, in the release of the spermatozoa from the germinal epithe-

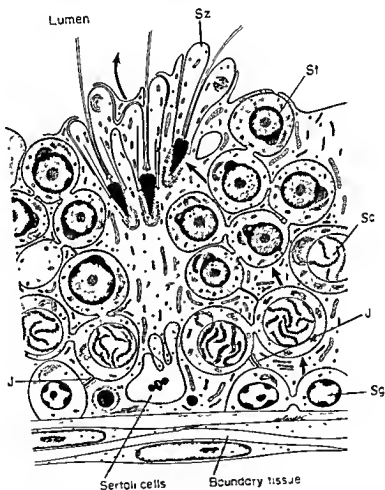


FIG. 6. A diagram of the arrangement of the germinal cells and the Sertoli cells in the seminiferous tubule. The diploid spermatogonia (Sg) are contained between the Sertoli cell cytoplasm and the boundary tissue of the tubule. The spermatocytes (Sc) and early spermatids (St) are sandwiched between pairs of Sertoli cells, on the luminal side of the specialized junctions (J) between adjacent Sertoli cells. The late spermatids (Sz) are embedded in the luminal surface of the Sertoli cell cytoplasm, until they are finally extruded into the lumen and leave the testis. Reproduced by permission of the publishers from Fawcett (18).



lium, as the heads of the spermatozoa are extruded into the lumen while the Sertoli cell retains most of the spermatid cytoplasm. A steroidogenic function has also been proposed for the Sertoli cells, but the evidence for this is extremely indirect. It is, however, quite likely that the cells inside the tubules transform one steroid into another, as many tissues do, without synthesizing steroids *de novo*. (15, 16). One very important function which has definitely been ascribed to the Sertoli cells is the synthesis of a specific androgen-binding protein. This protein facilitates entry of testosterone from the interstitial tissue into the tubules, and its production is under the control of FSH (24). The production of this protein has recently been demonstrated in isolated cultures of rat Sertoli cells (20).

In immature or hypophysectomized rats, FSH has also been shown to be bound to the seminiferous tubules and to have a number of metabolic effects there, some effects being evident within minutes of injection. As these effects can also be demonstrated in testes of rats treated *in utero* so that they contain no germinal cells, it seems likely that the primary site of action of FSH is the Sertoli cell (36).

The Sertoli cells are probably also the source of the fluid secreted into the lumina of the seminiferous tubules. This fluid can be seen if a portion of the lumen of a tubule is filled with colored oil. As secretion continues, the fluid breaks up the column of oil. The fluid can be removed for analysis; it is low in sodium and chloride and high in potassium and probably in bicarbonate. The spermatozoa are presumably carried from the tubules by the fluid into the rete testis and then into the epididymis. Fluid collected from the rete testis, however, is quite different from the fluid secreted in the tubules. The fluid normally present in the tubules is intermediate in composition between rete testis fluid and that secreted in the tubules (45, 46). It has therefore been suggested that there are two fluids secreted, one in the tubules and another, in much larger quantities, in the rete, and these are mixed in the tubules during the expulsion of the spermatozoa. It is not known whether this is due to an ebb and flow effect or whether fluid is drawn in at one end of the tubule and expelled from the other. The two-fluid hypothesis is supported by measurements of the concentrations of spermatozoa in the various fluids.

Rete testis fluid is also different in composition from blood plasma or testicular lymph, with regard to its inorganic (45, 46) and organic constituents. There is very little protein, and practically no glucose in rete testis fluid, but there are high concentrations of certain other substances such as inositol and glutamic acid, and appreciable amounts of testosterone (40, 41). It is also being used as a source for a nonsteroid hormone, "inhibin," which is produced by the seminiferous tubules and which regulates the production of FSH (43).

## E. THE BLOOD-TESTIS BARRIER

The fact that rete testis fluid and seminiferous tubular fluid differ in composition from blood plasma and testicular lymph suggests that substances do not diffuse freely in and out of the seminiferous tubules. Such a conclusion was also suggested by studies with injected dyes which stained most tissues, but did not stain the contents of the seminiferous tubules or the brain. The finding with the brain was avidly received and led to the important concept of the blood-brain barrier, but the observations on the testis were ignored for many years. Eventually, the quantitative significance of the blood-testis barrier was established by infusing a series of marker substances into the bloodstream in such a way as to maintain a constant concentration in the blood plasma while testicular lymph and rete testis fluid were collected. The rete testis fluid was taken to represent the fluid inside the tubules while lymph was thought to represent the fluid from the interstitial spaces. All the markers used passed readily from blood into testicular lymph, suggesting that there was little or no restriction of passage through the capillary wall, unlike the brain. However, there was a great variation in the rate at which the markers appeared in rete testis fluid. Some markers, like tritiated water, appeared almost as quickly in rete testis fluid as in testicular lymph, but others, including some quite small molecules like the chromium salt of ethylenediaminetetraacetic acid (Cr-EDTA) do not appear at all in rete testis fluid in experiments lasting up to 6 hours. Glucose enters the tubules by facilitated diffusion. Proteins, including the gonadotropins enter very slowly. Testosterone enters rapidly, but other steroids enter more slowly and cholesterol is virtually excluded (46, 44).

The assumption that rete testis fluid mirrors the fluid inside the tubules may not be entirely justified and because the volume of fluid secreted in the tubules is probably much less than that secreted in the rete, measurements of the entry rate into rete testis fluid may give very misleading results if the rate of passage through the wall of the rete is very different from that through the tubule wall. Therefore, other indirect techniques have been developed to study the entry of substances into the fluid in the tubules themselves, because the samples which can be removed by micropuncture for analysis are too small for the sort of experiment described above. Basically the same results were obtained as with rete testis fluid, and it would therefore appear that the permeabilities of the retial and tubular epithelia are similar. On the other hand, there is evidence that the rete is more permeable to dyes and proteins than the tubule, and the immunological reaction which occurs when an animal is injected with his own spermatozoa or testis homogenized in Freund's complete adjuvant,

seems to begin at the rete testis and spread from there along the tubules.

The blood-testis barrier in the seminiferous tubules can be morphologically localized at one or two sites in some species but these studies have not yet been performed in the domestic animals. In rodents, the myoid cells form end-to-end junctions with one another and these restrict the entry of most electron-opaque markers at most sites. However when the markers do pass the myoid cell layer, which they do at a small proportion of sites in rodents, but almost everywhere in monkeys and fowls, they penetrate around the spermatogonia and between adjacent pairs of Sertoli cells as far as the specialized junctions between them (Fig. 6). The markers stop there and do not make contact with the spermatocytes, spermatids, or spermatozoa.

It should be emphasized that all the markers used for electron microscopy are fairly large molecules, and smaller molecules may have a different route of entry, e.g., through the Sertoli cells. However, it is clear that the cells in the process of meiosis and their haploid offspring do not have ready access to the blood plasma or lymph and must rely on what is passed to them by the Sertoli cells.

The blood-testis barrier develops just before puberty. Its effectiveness seems to depend on temperature in a rather curious fashion, and is decreased after administration of cadmium salts and immunization of the animal with a mixture of testis homogenate and Freund's complete adjuvant.

The significance of the blood-testis barrier is not fully understood, but presumably it is concerned with creating the most favorable conditions for spermatogenesis, or more specifically for the meiotic division inside the tubules. The barrier would also limit the access of gonadotropins to the germinal epithelium, particularly the spermatocytes onward. This makes it more likely that these hormones, in particular FSH, exert their primary tubular effect on the Sertoli cells, to which access would be much easier, although still somewhat restricted. Since testosterone penetrates so readily, it seems most unlikely that there would need to be *de novo* synthesis of this compound inside the tubule, although if testosterone is transformed into other nonpermeable compounds inside the tubules, the barrier would serve to retain the metabolites inside the tubule.

The barrier also has important immunological consequences. It segregates the germinal cells, particularly the haploid cells, from the body's immunological system which is the reason why these cells are treated as foreign cells when injected into the body. The barrier also prevents naturally occurring antibodies, which react with spermatozoa and haploid germinal cells, from reaching these cells under normal conditions (42, 44, 46).

## F. TESTICULAR SPERMATOZOA

When the spermatozoa leave the testis, they are far from mature. They are virtually immobile, incapable of fertilizing eggs, and still contain a small globule of cytoplasm (called the cytoplasmic or kinoplasmic droplet) around the midpiece just behind the head. Their composition and metabolism are quite different from those of ejaculated spermatozoa (45).

Unfortunately, almost all the studies on the metabolism of testicular spermatozoa have been done in synthetic media resembling plasma more closely than rete testis fluid and therefore it is difficult to relate these studies to the situation *in vivo*. However under almost all conditions so far studied, testicular spermatozoa are quite different metabolically from epididymal or ejaculated spermatozoa. The latter convert a high proportion of glucose to lactate, even under aerobic conditions, whereas testicular spermatozoa convert a larger proportion to carbon dioxide and amino acids and to intracellular inositol, lipids, carboxylic acids, and a number of unidentified perchloric extractable compounds (45, 48).

Recently, however, ram and boar testicular spermatozoa have been incubated in a saline medium that resembled rete testis fluid in its ionic composition and that was buffered with carbon dioxide bicarbonate. The addition of glucose, inositol, glutamate, or ascorbate (all compounds present in high concentrations in rete testis fluid) to this medium had very little effect on oxygen uptake, although the same spermatozoa incubated in rete testis fluid have a significantly higher oxygen uptake than when incubated in the saline medium (13). It is also worth noting that testicular spermatozoa do not metabolize glucose via the pentose cycle. In this, they resemble ejaculated spermatozoa, but differ from the whole testis which has a relatively active metabolism of glucose via the pentose cycle (45). Testicular spermatozoa contain appreciably more lipid than epididymal spermatozoa, and are capable of synthesizing lipid from glucose at a much greater rate than ejaculated sperm. These lipids may be important sources of metabolic substrate for the spermatozoa during their sojourn in the epididymis (45).

## III. The Epididymis

### A. STRUCTURE

When the spermatozoa leave the testis, they pass through the efferent ducts into the epididymis. This is a compact fibrous organ closely applied to the posterior or superior border of the testis, and it consists basically

of a single very convoluted duct. Usually the epididymis is divided anatomically into head, body, and tail (*caput, corpus, and cauda epididymidis*); the head and tail are the enlarged portions at the two ends, the body the thinner part in between (Fig. 5). However, the microscopic anatomy of this duct varies appreciably along its length, as the function of the epididymis changes. Complicated anatomical subdivisions have been proposed but it is probably sufficient to consider that there are three main segments, namely initial, middle, and terminal segments. The initial segment has a high epithelium and a narrow lumen containing very few sperm; it is probably involved in the reabsorption of most of the fluid leaving the testis. The middle segment has a slightly lower epithelium and a wider lumen with many spermatozoa; in this segment the spermatozoa "mature" (see below). The terminal segment has a low epithelium and a very wide lumen packed with spermatozoa; this is the storage site for the spermatozoa awaiting ejaculation. Unfortunately this subdivision based on histology and function does not always coincide with the anatomical subdivision. In the ram and bull, the head of the epididymis contains the initial segment and part of the middle segment, although the boundary between the latter and the terminal segment does coincide with the boundary between body and tail. In other species the situation can differ (21).

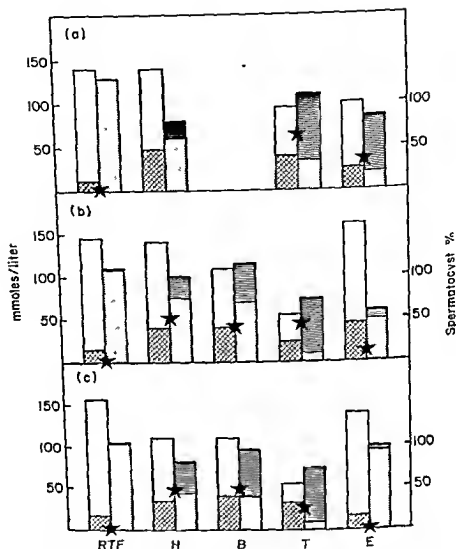


FIG. 7. A graph of the sodium (clear columns), potassium (diagonal light hatching), chloride (dots), and glycerylphosphorylcholine (close hatching), concentrations in the seminal plasma, and the spermatozoa (stars) in fluid from the rete testis (RTF), head (H), body (B), and tail of the epididymis (T), and ejaculated semen (E) of (a) rams, (b) bull, and (c) boar. The epididymal samples were from slaughterhouse material; the rest from conscious animals. Based on values from Crabo (8a), Mann (32-35), White (51) and Setchell (40 and unpublished).

### C. SPERM MATURATION

The transition from the immotile, infertile spermatozoa which leave the testis to motile, potentially fertile spermatozoa occurs at a fairly sharply defined area of the epididymis (at least in rabbits, hamsters, and rats in which most of the work on this topic has been done; this point has not yet been studied in the larger domestic animals). In the rabbit, the ability of the sperm to fertilize is acquired in the distal half of the body of the

epididymis, although the proportion of fertile sperm in this zone may still be less than in the tail of the epididymis or the ductus deferens. The role of the epididymis in the development of fertility of the sperm was established in experiments in which the spermatozoa were retained in various parts of the epididymis with ligatures. Those held in the most proximal parts of the epididymal duct never became fertile; those held just proximal to the zone where fertility is usually acquired, did become fertile.

The capacity for full progressive motility also develops in the corpus epididymidis, but in contrast with fertility, motility can develop in rabbit spermatozoa when they are held by ligature in any part of the epididymis; however, spermatozoa held in the cauda retain the capacity for full motility for 30 to 60 days, whereas the motility of those held in the caput persists for only a few days. Thus, there appears to be a separation possible of the development of motility from that of fertility.

The survival of spermatozoa passing through the epididymis depends on normal androgen production by the testis; castration or hypophysectomy rapidly causes death of the spermatozoa but injections of testosterone can keep them motile and fertile. Other androgens such as  $5\alpha$ -androstane- $3\alpha,17\beta$ -diol which have a lower androgenic potency than testosterone on the accessory glands may be more effective than testosterone in maintaining the spermatozoa in the epididymis. Similar effects can be demonstrated in spermatozoa held in specific parts of the epididymis by ligatures and again the capacity for fertility and motility can be dissociated. The effective androgen is probably blood-borne, not carried in the rete testis fluid, as ligation of the efferent ducts is without effect on the spermatozoa already in the epididymis (38).

A number of morphological changes also occur in the spermatozoa as they pass along the epididymis, the most obvious being the movement of the kinoplasmic or cytoplasmic droplet along the midpiece and its eventual loss. In the stallion and bull this loss occurs in the middle of the caput; in the ram and boar it occurs immediately distal to the caput. The droplet is a remnant of spermatid cytoplasm and contains, like the acrosome of the sperm, appreciable amounts of many lysosomal enzymes. The shape of the acrosome in some species also changes as the sperm passes down the epididymis. There are also subtle changes in the sperm plasma membrane, and the chromatin of the nucleus of the spermatozoa also becomes more stabilized due to the formation of —S—S— linkages (3).

#### D. PASSAGE OF SPERMATOZOA THROUGH THE EPIDIDYMIS

The spermatozoa leave the testis in the rete testis fluid which carries them into the caput epididymidis. There most of the fluid is reabsorbed and, as their further movement along the duct seems to be unaffected by

ligation of the efferent ducts, it seems likely that contractile activity of the muscle surrounding the epididymal duct is responsible for their movement within the epididymis.

Using techniques similar to those used for timing spermatogenesis (see Chapter 8), it has been shown that transit time of the spermatozoa through the epididymis is about 13 days in the ram, about 11 days in the bull, 9–14 days in the boar, and 8–10 days in the rabbit. There is a slight decrease in the transit time in animals which are ejaculating frequently but this reduction is usually only about 10 to 20% of the total transit time (3).

#### E. FATE OF UNEJACULATED SPERMATOZOA

There are two schools of thought on the fate of unejaculated spermatozoa. One suggests that the spermatozoa are destroyed and reabsorbed in the male tract, and the other suggests that they are extruded into the urethra and then washed away by the urine. The main evidence for the former view comes from failure to find enough spermatozoa in carefully collected urine samples to account for calculated sperm production rates. Nevertheless, it is strange, in a sexually inactive male, that no sign of the degenerating spermatozoa can be found in the epithelium of the epididymis and ductus deferens. On the other hand, there is now good quantitative evidence for the latter view in rams. If the spermatozoa in the urine are prevented from agglutinating, thereby becoming difficult to count accurately, the output in the urine over a period does correspond with the number emerging from a catheter in the rete testis. However, the number in the urine is highly variable from day to day, and there is still a possibility that quantitative differences may exist between species.

The effects of ligation of the ductus deferens in the ram suggest that spermatozoa in this species do not degenerate in the lumen of the epididymis; degeneration only occurs when the duct ruptures to form a spermatocele. Similarly, in the bull, there was a fibrotic enlargement of the ductus deferens at the site of vasoligation where most of the spermatozoa accumulated and probably were absorbed, although it was not possible to tell whether rupture of the duct had occurred (3).

### IV. The Accessory Glands

#### A. ANATOMY

The main accessory sex glands in the male are the ampullae, the seminal vesicles, the prostate, and Cowper's glands (also known as the bulbo-



urethral glands) (Fig. 8) all of which produce secretions which contribute to ejaculated semen. There are also other urethral glands (Littre's glands) and preputial glands, but little is known of their function or the composition of their secretion.

The ampullae appear as dilations of the urethral end of the ductus deferens. They are particularly well developed in the stallion but absent in the boar.

The seminal vesicles are situated on either side of the neck of the bladder and were so named because they were thought to act as a store for "semen" from the testis. Although this is almost certainly not correct, it was a reasonable deduction in man, as in this species the ductus def-

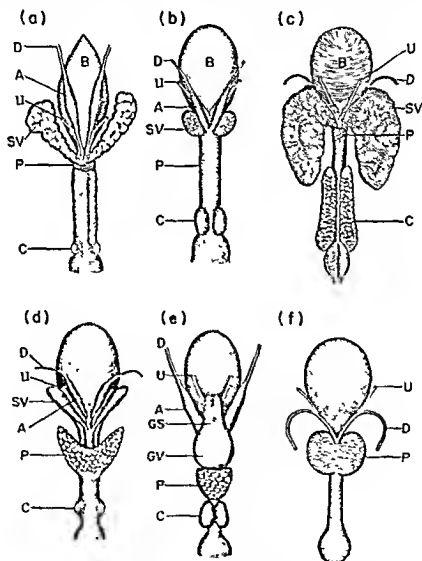


FIG. 8. Diagrammatic view of the dorsal aspect of the accessory glands of (a) bull, (b) ram, (c) boar, (d) stallion, (e) rabbit, and (f) dog. SV, seminal vesicle; P, prostate, in the ram inside the urethral muscle; C, Cowper's glands; A, ampulla of the ductus deferens (D); U, ureter; B, bladder; GS, glandula seminalis; GV, glandula vesicularis (a), (c), and (d) after diagrams in Fekstein and Zuckerman (11), (f) after diagrams in Ackerknecht (1), and (b) and (e) from fresh specimens.

erens and the seminal vesicles open into a common "ejaculatory duct." This duct is not found in any of the domestic animals, in which each ductus deferens and seminal vesicle open separately into the urethra. The dog has no seminal vesicles. In the bull and boar, they are very large and contribute a large fraction of the ejaculate. In the rabbit, there are two organs with a common duct, the "glandula seminalis" and the "glandula vesicularis," which are thought to be homologous to the seminal vesicles, of other mammals.

The prostates lie caudal to the seminal vesicles, close to where the bladder joins the urethra. In the domestic ruminants, the prostate is inconspicuous, as it is disseminate and does not penetrate the muscular covering of the urethra. In the horse and pig, the gland is located both within the muscle and outside as a discrete "lobe." In the dog, the prostate is the principal accessory gland and, as in man, it is a relatively large, prominent organ which completely encircles the urethra.

The Cowper's or bulbourethral glands are associated with the caudal portion of the urethra and drain into the pelvic urethra in its bulbar region. In the bull, ram, and stallion they are comparatively small; they are absent in the dog, but in the pig they are large cylindrical bodies, which produce a very viscous secretion most important in the process of "gelation" of boar semen after ejaculation (11, 32).

## B. SECRETIONS OF THE ACCESSORY GLANDS

In most species, the secretions of the various accessory glands constitute the bulk of the ejaculated semen. Certain of the characteristic constituents of seminal plasma are contributed by certain individual glands. These substances include fructose, citric acid, ergothioneine, inositol, and prostaglandins. A knowledge of the origin of these substances is very important in assessing the function of the individual glands from the composition of the semen. A summary of the available information on the origins of the specific components of seminal plasma, based mainly on the work of Mann and his colleagues is given in Table I. Fructose is formed in the tissue from blood glucose via sorbitol; the chemistry of formation of the other compounds is less well understood but they all seem to be synthesized in the glands concerned. Glycerylphosphorylcholine is produced not in the accessory glands, but in the epididymis.

The function of all of these glands is dependent on the secretion of testosterone by the testis, and the concentration of the various specific constituents in the semen is closely related to testosterone secretion (32-35). The Cowper's glands of wethers (castrate male sheep) are also stimulated to hypertrophy by estrogenic compounds found in subterranean

TABLE I

The Origins of Characteristic Substances Found in Semen<sup>a, b</sup>

	Bull	Ram	Goat	Boar	Stallion	Dog	Rabbit
Seminal vesicle	FCi(e-)	FCPr	F	FCIE	C	Gland absent	FC
Prostate				c		P(c-, f-)	Fc
Cowper's gland				S(e-, i-)		Gland absent	c
Ampulla	fc		f	Gland absent	Ei(f-, c-)		FC

<sup>a</sup> Based on data from Mann (32, 33).<sup>b</sup> F, fructose; C, citric acid; I, inositol; E, ergothioneine; Pr, prostaglandins; P, proteolytic enzymes; S, sialoproteins. Capital letters indicate high concentrations, lower case letters indicate moderate to low concentrations, lower case with minus sign in parenthesis indicates substance virtually absent.

clover, sometimes leading to obstruction of the urethra and consequent clinical problems (37). Secretion by the dog prostate is stimulated by injections of pilocarpine, suggesting that this secretion is normally under parasympathetic control (32).

## V. Semen

The ejaculated semen comprises the spermatozoa suspended in the seminal plasma. The volume of the ejaculate ranges from 1 ml in the ram to as much as 500 ml in the boar, and the concentration of the spermatozoa varies inversely.

### A. SPERMATOZOA

The structure of a typical ejaculated mammalian spermatozoon is illustrated in Fig. 9. The head consists mainly of highly condensed chromatin, its anterior half covered by the acrosome. In the domestic mammals the acrosome is a reasonably inconspicuous structure, but in some rodents it takes quite extravagant forms. It consists of an inverted membranous sac containing a specific lipoglycoprotein complex which includes a number of enzymes such as hyaluronidase and acrosin. The former breaks down mucopolysaccharides and is probably involved in dispersing the cumulus oophorus. The latter is involved in the penetration of the spermatozoa through the zona pellucida of the egg. Techniques have recently been developed for removing the acrosomes from the spermatozoa without damage (5). The caudal face of the head is indented to allow attachment of the midpiece and tail. These are formed around a central struc-

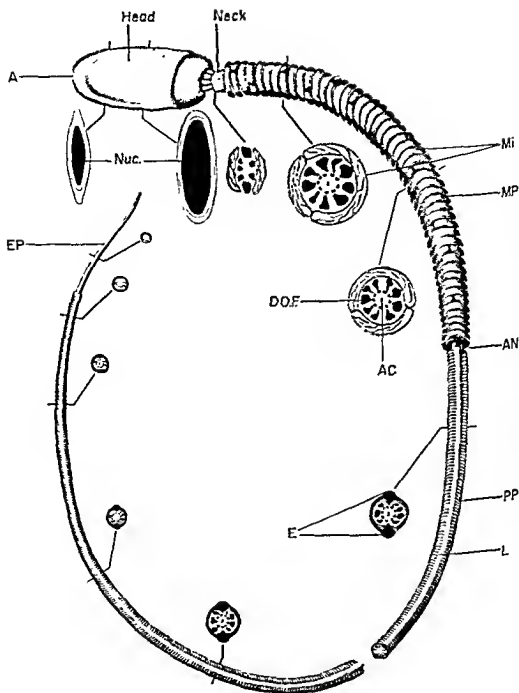


FIG. 9. Diagram of an ejaculated mammalian spermatozoon with cross sections at various levels, as indicated. A, acrosome, Nuc, nucleus; Mi, mitochondria, which are helically arranged around the midpiece (MP); AC, axonemal complex of two single central and nine pairs of peripheral microtubules; DOF, nine dense outer fibers, one of which corresponds to each of the nine peripheral pairs of microtubules; AN, annulus marking the end of the midpiece and mitochondria, and the beginning of the principal

ture of  $9 + 2$  filaments, a formation which is common to many cilia in a wide range of organisms. Outside each filament is a "dense outer fiber" which may have some contractile function. The tail is encased in a fibrous sheath (39), but the midpiece is characterized by the mitochondria which provide the energy for the motility of the spermatozoon and which are helically arranged around the fibers of the tail (Fig. 9). The tail of a bull sperm beats at a rate of about 10/second and the sperm can progress at about  $100\text{ }\mu\text{m}/\text{second}$  in physiological saline but rather less in cervical mucous (32, 4). The nucleus contains a haploid set of chromosomes composed primarily of deoxyribonucleic acid (i.e., half that in somatic cells), combined with basic proteins to form deoxyribonucleoproteins. Because the X and Y chromosomes are separated during the meiotic division, the spermatozoa should form two populations, "male-producing" and "female-producing" but claims that these two populations have been separated have not so far been substantiated.

The mature spermatozoa contain very little cytoplasm (the remains of the spermatid cytoplasm which form the cytoplasmic or kinoplasmic droplet left attached to the spermatozoa when it leaves the germinal epithelium, is lost from the sperm during its passage through the epididymis). Therefore, it is not surprising that the sperm contain very little ribonucleic acid. However, the spermatozoa do contain appreciable amounts of neutral lipids and phospholipids, some of which may be utilized by the spermatozoa as energy reserves in the epididymis. It is interesting that in bull and ram sperm, a high proportion of the phospholipid is choline plasmalogen (also known as phosphatidylcholine) which differs from lecithin (phosphatidylethanolamine) in having a vinyl ether linkage attaching one of the fatty acid side chains to the glycerol skeleton (32-35).

The metabolism of spermatozoa has been studied in some detail. Bull and ram spermatozoa are capable of breaking down fructose or glucose rapidly to lactic acid under anaerobic conditions, but boar and stallion sperm do so at a much slower rate; sperm from all species can oxidize these sugars to carbon dioxide under aerobic conditions. Anaerobic fructolysis by bull and ram sperm is closely correlated with motility, but the relationship with fertility is less certain. A number of other substrates can also be utilized including lactic acid, pyruvic acid, acetic acid, glycerol, and sorbitol.

The motility of spermatozoa is highly correlated with the intracellular

---

piece (PP). In the first part of the principal piece, dense outer fibers 3 and 8 terminate and are replaced by inward extensions (E) of the longitudinal columns (L) of the fibrous sheath. The fibrous sheath stops at the junction of the principal piece and the endpiece (EP). Reproduced by permission of the publishers from Fawcett (17).

TABLE II  
Some Details of the Composition of Semen of the Domestic Animals\*

	Bull	Ram	Goat	Boar	Stallion	Dog	Rabbit
<b>Semen</b>							
Volume (ml)	2-10	0.5-2	0.5-2.5	150-500	20-300	2-15	0.4-6
Dry weight (%)	9.5	14.8		4.6	4.3	3.8	
Sperm concentration ( $\times 10^{-6}/\text{ml}$ )	300-2000	2000-5000	1000-5000	25-350	30-800	60-300	50-350
Spermatozoa (%)	10	33		2	3	6.1-7.0	6.59-7.5
	6.48-6.99	5.9-7.3		6.85-7.9	6.2-7.8	1.011	
pH	1.035				1.013		
Specific gravity							
<b>Seminal plasma</b>							
Protein (gm/100 ml)	3-8				<1	2.1	40-150
Fructose (mg/100 ml)	120-540	150-600		20-40	20-60	<1	80
Sorbitol (mg/100 ml)	10-136	26-120		36-325	8-53		
Citric acid (mg/100 ml)	357-1000	137					
Ascorbic acid (mg/100 ml)	8.7	5		380-610	19-47		
Inositol (mg/100 ml)	25-46	10-15	Absent	6-30	3.5-13.7		
Ergothioneine (mg/100 ml)	Trace						
Glutamic acid (mg/100 ml)	35-41						
Glycerol-phosphorylcholine (mg/100 ml)	110-500	76	1400-1600(WS) <sup>b</sup>	110-240	40-110(WS) <sup>b</sup>	180(WS) <sup>b</sup>	215-370(WS) <sup>b</sup>
Sodium (mmoles/liter)	117	78		122	114	114	
Potassium (mmoles/liter)	44	23		16	26	8.1	
Calcium (mmoles/liter)	9.3	1.9			6.5	0.35	
Magnesium (mmoles/liter)	3.4	2.4			3.8	0.25	
Chloride (mmoles/liter)	49	18		96		152	
Bicarbonate (mmoles/liter)	7.1(WS) <sup>b</sup>	7.1(WS) <sup>b</sup>				2.9(WS) <sup>b</sup>	
$\alpha$ -Mannosidase (units/ml)	400	50					
$\beta$ -N-Acetylglucosaminidase (units/ml)	15,000	16,000					

\* Based on data from Mann (32, 33).

content of cyclic AMP (adenosine-3',5'-cyclic monophosphate), and this important coenzyme is readily lost from the sperm during storage or if they are damaged. By contrast, the levels of NAD (nicotinamide adenine dinucleotide), another important coenzyme, do not decrease during limited storage, although the ratio of oxidized to reduced forms can be readily altered.

The production of organic peroxides during storage may also exert important toxic effects on the spermatozoa (32-35).

## B. SEMINAL PLASMA

As already indicated in the section on the individual accessory glands, seminal plasma contains a number of substances in much higher concentrations than occur elsewhere in the body. It is curious that many of these substances are characteristic of plants rather than animals! Those studied so far include fructose, citric acid, inositol, ergothioneine, glycerylphosphorylcholine, glutamic acid, and certain enzymes. The concentrations found in the different species are given in Table II. It should be emphasized that the concentrations are dependent on the rate of testosterone secretion by the testis, and may vary in different samples of semen, or in different portions of the one ejaculate, if the contribution of the different accessory glands varies. Ejaculation is not instantaneous and a "split-ejaculate" method can be used to collect fractions of the ejaculate in a number of species including boar and stallion, and the bull if electroejaculation is used. For example, in the stallion, pre-sperm, sperm-rich, and post-sperm fractions can be collected. The pre-sperm fraction is watery and contains few sperm and low concentrations of ergothioneine and citric acid. The sperm-rich fraction also contains most of the ergothioneine which comes from the ampullae. The post-sperm fraction is mainly seminal vesicle secretion, and contains few sperm but a high concentration of citric acid. There is often a later fraction which drips from the stallion's penis after dismounting, but this is very low in spermatozoa and not at all representative of the whole ejaculate (32-35).

However, from a study of a composition of the whole ejaculate or the appropriate fractions a great deal can be learned about the sperm and hormone production by the animal. Semen collection has the tremendous advantage that it does not require any surgical intervention and can be used repeatedly on one animal.

## ACKNOWLEDGMENTS

I am particularly grateful to Professor D. W. Fawcett, Department of Anatomy, Harvard Medical School, Boston for allowing me to reproduce his illustrations as

Figs. 3, 6, and 9, to Mr. J. R. Fuller for the original drawings of Figs. 1, 2, 5, and 8, and to Professor T. Mann, Unit of Reproductive Physiology and Biochemistry, University of Cambridge for reading the manuscript and making many helpful suggestions.

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# 10 Artificial Insemination

W. R. Gomes

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## I. Introduction

Although the use of artificial insemination has been slowed by both rational and irrational arguments, no other new technique has been welcomed with so much approval throughout most of the world. Used for mating of about 20% of the world's cattle and millions of sheep, goats, swine, and horses (18), artificial insemination may be the best example in agriculture of rapidly applying basic laboratory research data to widespread industrial practice.

In this chapter, the physiological aspects of semen collection, preservation, and insemination will be summarized for several species. More extensive discussions and more inclusive reference lists will be found in many recent reviews on specific species or topics (41, 43, 49, 54, 69, 87, 88, 110, 112, 132, 141).

## II. Collection of Semen

Realization of the maximum benefits of artificial insemination depends upon the collection of maximal numbers of viable sperm cells at frequent intervals from genetically superior males. This involves equipment and procedures which can be used repeatedly while maintaining libido in the male. Collection techniques and frequencies which harvest the largest numbers of sperm over the longest period of time are desirable.

### A. GENERAL PRINCIPLES AND PROCEDURES

During semen collection, an area should be provided that is safe for the handler, yet which allows the male freedom from excessive restraint. This may be accomplished with posts or rails when handling bulls (141), wearing hard hats (41), and providing good underfooting for the animal. Surroundings should be relatively quiet and free of distractions. Live mounts, such as an estrous female or an estrogen-treated female may be necessary, but many bulls, rams, and stallions, and most boars can be trained to mount a dummy (Fig. 1), a castrated male, or even another stud animal. Live mounts should be carefully restrained to minimize movement, but use of a flexible stanchion or even a horse collar for cows (141) may help to prevent injury.

#### 1. Artificial Vagina

The foundation for the rapid expansion of artificial insemination has been the use of the artificial vagina. The artificial vagina is the method of choice for collecting semen from bulls, stallions, rams, and many other species (41); it has been successfully used for collecting from boars (126) and dogs (52).

Although the size and shape of the artificial vagina vary among species and several modifications exist from the basic construction (41), all are similar in design to the one illustrated in Fig. 2. The water jacket of the collecting unit is filled to a temperature and pressure preferred by the species (43, 44) and, if known, by the individual. The inner opening of

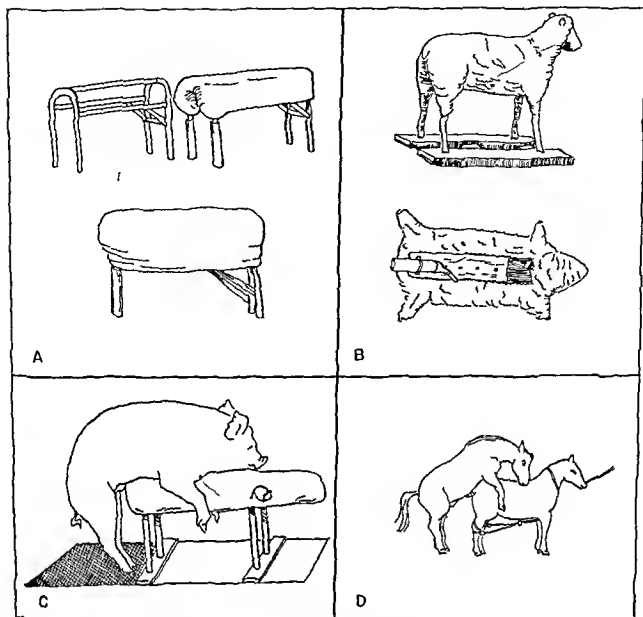


FIG 1 Dummy and live mounts used for semen collection (adapted from reference 44) (A) Top, dummy cow constructed from pipe (left) completed padded dummy (right) Bottom dummy mare (B) Dummy ewe with artificial vagina secured for semen collection (C) Bore mounting dummy on slip proof mat (D) Stallion mounting hobbled restrained and tail bindaged mare

the artificial vagina is lubricated to provide comfortable passage of the penis. The collecting tube may be calibrated to allow rapid estimation of semen volume.

## 2 Electroejaculation

When males are not trained to serve the artificial vagina, or suffer from injury, or infirmity, or when conditions are not conducive to normal mounting and ejaculation, electroejaculation provides a method for collecting healthy semen of normal viability. Newer models of electroejacu-

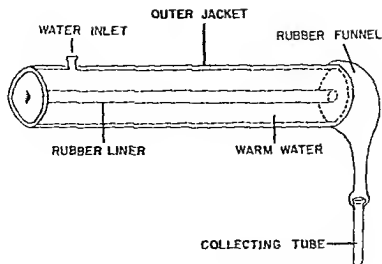


FIG. 2. Artificial vagina used for collecting semen.

lators generally employ a single rectal probe with bipolar electrodes. Rhythmic application of electrical stimulation to the male leads to secretion from the sex accessory glands and, finally, to ejaculation. Very good results have been obtained with electroejaculation of bulls, rams, and other species (53), but the procedure results in discomfort and lowered semen quality when used in boars (44) and dogs (23).

Although fertility of semen collected by electroejaculation has compared favorably with that collected using the artificial vagina, recent evidence suggests that ram sperm collected by electroejaculation are more susceptible to cold shock and less likely to survive freezing and thawing (124).

### 3. Other Methods

As mentioned above, boar semen may be collected using an artificial vagina; however, significantly more sperm per ejaculate were collected using the gloved or bare hand to grasp the penis of the ejaculating male (71, 102). Since this method is simpler and less expensive and since boars (or collectors) are more easily trained to accept it, the "gloved hand" technique appears preferable for collecting boar semen.

Masturbation is the preferable method of collecting semen from dogs (19, 84), chickens (41), turkeys (20), and geese (177).

Samples of semen can be collected by massaging the ampullae of bulls (141), by collecting semen from mated females, or by killing the male and stripping the cells from the reproductive tract [as has been done with the rat and the elephant (66)]. These techniques frequently lead to con-

taminated samples with disproportionate contributions from the sex accessory glands.

## B. EFFICIENCY OF SEMEN COLLECTION

The ultimate benefits of artificial insemination would be realized if all of the sperm cells produced by superior sires could be collected and utilized for insemination; however, many sources of sperm losses exist, including losses before collection, losses during collection and handling, and cell attrition during processing and storage.

### 1. Daily Sperm Output

Using histological and cytological methods to determine the cell types present in testis tissue samples or homogenates, investigators have calculated the daily sperm production in several species (6). More recently, insertion of a cannula into the rete testis has allowed others to directly measure the daily exodus of sperm from the testes of bulls (7, 170) and rams (169). If the surgical insertion or the presence of such a cannula does not alter the rate of sperm production, this method offers the ultimate in accuracy for determining daily sperm production rates (6).

When sperm are collected at an optimal frequency (the fewest collections per week that result in a maximal number of cells collected) for a period of time, the daily sperm output can be directly measured. As shown in Fig 3, sperm output was increased in the stallion by increasing the number of weekly ejaculations from 1 to 3, but additional collection periods were without effect (116).

Table I summarizes current estimates of daily sperm production rates, daily sperm output, and the efficiency of sperm output for a number of species. When estimates of sperm production rates were made from histological samples, it appeared that preejaculation losses of sperm were very large, especially in cattle. However, more recent data on sperm collected by rete testis cannulas (7, 169, 170) suggest that sperm production rates are lower than previously reported. Indeed, the data of Amann *et al.* (7) suggest that negligible numbers of sperm are absorbed by the bovine epididymis and daily collection of semen accounted for most of the sperm produced.

### 2. Effects of Sexual Preparation

Restraint of bulls or false mounts will increase numbers of motile sperm in the bull ejaculate by as much as 50% (141). Hafs *et al.* (59)

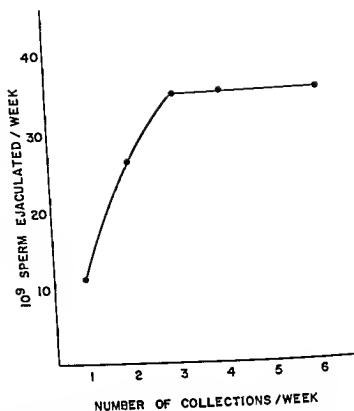


FIG. 3. Numbers of sperm collected from stallions with different frequencies of collection (drawn using data from reference 116).

TABLE I

Daily Sperm Production Rates, Sperm Output, and Collection Efficiency for Farm Animals\*

	Daily sperm production (DSP) (10 <sup>9</sup> cells/day) <sup>b</sup>	Daily sperm output (DSO) (10 <sup>9</sup> cells/day)	Collection efficiency [(DSO/DSP) 100] (%)
Dairy cattle	13.2 (6.5)	4.4	42 (85)
Beef cattle	4.0	1.3	33
Sheep	10.9 (7.8)	7.1	65 (90)
Swine	17.1	14.6	85
Horse	7.9	6.3	80
Rabbit	0.23	0.15	63

\* Calculated from data in references (6, 7, 48, 116, 169, and 170).

<sup>b</sup> Estimated from histological samples of testes. Recent data using cannulated testes to estimate DSP<sup>b</sup> for dairy bulls and sheep are summarized in parentheses.



showed that either restraint or false mounts would increase the number of cells ejaculated, and that three false mounts over a 10-minute restraining period could increase total sperm nearly threefold for dairy bulls (Fig. 4). Smaller increases were found for beef bulls (4). Large increases in total (261%) and motile (278%) cells were also found in the ejaculates of boars restrained up to 10 minutes compared with unrestrained boars (23).

The assumption that "teasing" procedures will be beneficial for other species cannot be made. A recent study by Pickett *et al.* (117) indicates that these procedures increased semen volume in stallion ejaculates at the expense of sperm concentration, resulting in no overall change in total sperm ejaculated.

### 3. Increasing Sperm Output

Since natural mating, ejaculation, and genital massage all cause increases in blood oxytocin in bulls (149), it has been suggested that oxytocin is, at least in part, responsible for the improvement in ejaculates following sexual stimulation. Improvements were found in sperm per

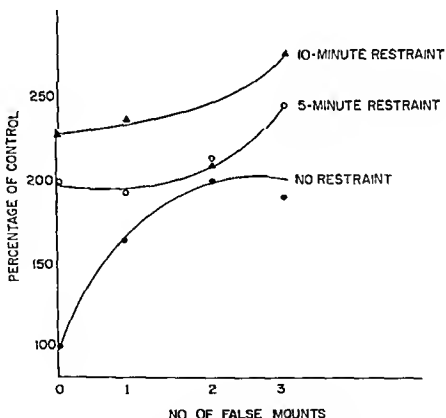


FIG. 4. Effect of restraint and false mounts on the number of sperm ejaculated by bulls, expressed as a percentage of the value (100%) for unrestrained, unteased bulls [adapted from the data of Hafs *et al.* (59)].

ejaculate when oxytocin was administered to bulls (94) or rams (74), but no attempt was made to determine whether effects of sexual stimulation and oxytocin were independent.

Hafs *et al.* (60) reported that prostaglandin  $F_{2\alpha}$  increased the movement of sperm into the ductus deferens of the rabbit and increased the number of sperm in the first ejaculate. Similar results were found in bulls whether the bulls were sexually stimulated by false mounts or not (54) if prostaglandin injections were given within 2 hours before collection. Pre-collection injections of prostaglandins  $F_{2\alpha}$  also increased cells ejaculated by stallions (25), but daily injections of the hormone had no beneficial effect on semen of rams; indeed, sperm concentrations and sperm numbers per ejaculate were decreased by the treatment (164).

#### 4. Sources of Sperm Cell Loss

As discussed by Amann (6) several factors may account for the difference between sperm production and maximum sperm output. These include losses in the artificial vagina, losses in discarded portions of the semen (i.e., the gelatinous fraction of boar or stallion semen), and losses in transfer from collecting to storage vessels. In addition, males may pass sperm in the urine or may spontaneously ejaculate, and in some species, epididymal resorption of cells may occur.

### III. Semen Evaluation

Obviously, the only valid measure of semen viability is to observe the fertility of inseminated females. However, a number of semen characteristics can be used to evaluate the general quality of the sample and to estimate the extent to which it may be extended. These characteristics vary widely between species (Table II) and may deviate considerably from the average of the species. The ultimate goal of quality tests is to accurately predict semen fertility using rapid, inexpensive techniques.

#### A. GENERAL EVALUATION

A properly collected semen sample should be near the volume characteristic of the species (Table II) and free of urine, hair, or foreign matter. Depending on the species, the color will vary from a creamy white (bull) to a watery grey (dog), but all should be opaque in appearance, indicating a high sperm concentration. In some bulls, yellowish semen is normal, but "off colors" may be indicative of contamination with urine, blood, or foreign material.

TABLE II  
Characteristics of Semen from Farm Animals

	Cattle											Turkeys
	Dairy	Beef	Sheep	Swine	Horses	Rabbits	Dogs	Cats	Chickens	Goats	Buffalo	
Volume (ml)	6	4	1	225*	60†	0.6*	5*	0.05	0.5	0.8	4	0.5
Sperm concentra- tion (10 <sup>9</sup> /ml)	1.2	1.0	3.0	0.2	0.15	0.5	0.3	1.5	3.5	2.4	1.0	7.0
Total sperm (10 <sup>9</sup> )	7	4	3	45	9	0.03	1.5	0.1	1.8	2.0	4	3.5
Motile sperm (%)	70	65	75	60	70	80	85	75	85	80	60	60
Morphologically normal sperm (%)	80	80	90	60	70	80	80	90	90	90	80	85
Ejaculates/week	4	4	20	3	3	6	3	—	3	20	3	3
Living cells/ insemination(10 <sup>4</sup> )	10 <sup>4</sup>	10 <sup>4</sup>	120	1200	100	25	100	5	60	60	10	125

\* Gel-free volume.

† See Section VI.C.

Motility is one of the most widely used tests for semen quality. The test, properly conducted, has been considered a good indicator of general sperm viability, but it must be recognized as only one factor in bovine fertility (43). Furthermore, motility may even be a poor measure of fertility in boars, rams, and stallions (41, 136). The proportion of motile and progressively motile cells is determined subjectively during microscopic evaluation of the sample. In some cases, objective evaluation of motility has been conducted (171) but use of such techniques is not widespread.

As sperm cells die, they become permeable to eosin. Therefore, this stain can be used to determine the ratio of live:dead cells in a sample (141). Stained slides may also be used to examine the morphology of sperm. Abnormalities of the head, midpiece, and tail may be due to defective mechanisms in the reproductive tract of the male or to changes caused during collection and processing (141). In recent years, changes in the acrosome of the sperm head have been related to sperm fertility (see Section III,B).

Concentration of sperm cells in the semen is estimated using a hemacytometer for initial or occasional samples, but use of a calibrated spectrophotometer is common for routine assessment of sperm numbers (43, 111, 141). Electronic cell counting equipment can also be used (70). Accurate determination of concentration, semen volume, and percentage live cells is essential in estimating the maximum dilution of sperm which can be prepared for artificial insemination.

Measurement of the metabolic activity of sperm cells after standardization for sperm concentration is positively correlated with cellular activity (43, 141). Glycolytic activity can be estimated by glucose uptake, fructolysis, or lactic acid production. Oxygen consumption, carbon dioxide production, or pyruvate oxidation can be measured directly or estimated indirectly (141). Buffering capacity, electric conductivity, resistance to cold shock, and *in vitro* livability also measure sperm vitality.

## B. PHYSICAL AND BIOCHEMICAL EVALUATION

Based on light and electron microscope studies, Saacke and his co-workers (131, 132, 134, 135) and Wells *et al.* (174) have suggested that alterations of the acrosome cap may reflect detrimental changes in the sperm cell. Increases in such alterations were found during sperm aging and injury (131, 134), with infrequent semen collection (174), and with incubation or freeze-thaw damage (135). In more extensive studies, Saacke and White (131) reported that the frequency of intact acrosomes in semen was highly correlated with fertility of bulls and of

individual semen samples. Fragmentary data suggest that a similar relationship may exist in boars (123) and rams (156).

Alterations in acrosomal membranes which occur with injury to sperm may also lead to the release of acrosomal contents, including enzymes. If this is so, an assay of the release of acrosomal enzymes into the seminal plasma should provide an early indication of damage sustained by spermatozoa during collection, processing, or storage. Foulkes and Watson (47), using laboratory tests, confirm that a relationship exists between seminal plasma hyaluronidase levels and measures of damage to bull sperm, but the relationship to fertility has not yet been tested. Similarly, Brown *et al.* (21) suggested that glutamic-oxalacetic transaminase levels increase in seminal plasma following freeze-thaw damage to bull, boar, and turkey semen, and Tash and Mann (163) reported a loss of cyclic AMP from cells following damage to ram, bull, boar, stallion, or chicken sperm.

### C. COMPETITIVE FERTILIZATION

By insemination of mixed ejaculates from two sources (heterospermic insemination) it is possible to compare the fertilizing ability of both samples based on their competitive performance within the same female tract. It is necessary that the source of the sperm cell which fertilizes each ovum be determined. As discussed by Beatty *et al.* (12), the ability to distinguish fertility differences between two males is improved by a factor of 170 to 340 when heterospermic as opposed to homospermic inseminations are used. Using homospermic inseminations, 170-340 times as many matings must be made, compared with assessing fertilizing ability in direct competition.

Heterospermic inseminations using breeds of different colors (11, 12, 33, 81, 99, 106, 115, 127), animals of different blood types (3, 6, 158), or chemical "marking" of sperm cells (13, 119, 121) as the basis for identifying "successful" sperm in competitive fertilization schemes have been used to evaluate semen from different males or different semen treatments using cattle, mice, rabbits, sheep, and swine (11, 90, 115). The combination of "marking" semen and investigation of early embryos for preferential sperm penetration (13, 119, 121) promises to be an important step forward for rapid evaluation of semen and processing techniques.

## IV. Preservation and Dilution of Semen

Seminal plasma contains sufficient environmental agents to maintain ejaculated sperm for relatively short periods of time. If sperm are to be

diluted and stored prior to insemination, the beneficial properties of the seminal plasma must be extended. In addition, a semen "extender" (which increases the number of inseminations possible and prolongs the viability of the sperm) must provide conditions which allow the sperm to withstand the unnatural conditions associated with preservation and artificial insemination. Salisbury and VanDemark (141) have summarized the criteria for a satisfactory extender. In general, an extender must (a) be of an osmotic pressure isotonic with spermatozoa, (b) provide a proper balance of minerals essential to the life of sperm cells, (c) provide an energy source for sperm metabolism, (d) protect sperm cells against cold shock, (e) provide buffering capacity against metabolic products, and (f) be free of, and protect sperm from, bacterial or infectious organisms which might prove harmful. In addition, semen extenders must contain cryptoprotective agents to protect the sperm cells during freezing.

#### A. COMPOSITION OF EXTENDERS (DILUENTS OR ADDITIVES)

Since Phillips (141) discovered the beneficial effects of egg yolk on extension and preservation of sperm fertility, many semen extenders for several species have utilized this ingredient. The primary benefit derived from fresh egg yolk is protection of the sperm cell against cold shock by yolk lipoproteins and lecithin (141).

Use of whole or skim milk in semen extenders also protects sperm against cold shock while satisfactorily maintaining fertility, provided the milk is heated prior to use (141). The milk protein, casein, has been established as the agent responsible for prevention of cold shock (107).

Although yolk or milk constituents may replace the whole product in semen extenders, virtually all extenders in use today contain egg yolk, milk, or a combination of the two, for prevention of cold shock.

✓ Buffering capacity is frequently provided by Tris-sodium citrate for yolk-containing buffers, but homogenized milk contains sufficient buffering agents in itself. Extenders have also utilized bicarbonate, potassium chloride or potassium tartrate, and various sugars to provide optimum osmolarity and buffering capacity (41).

Semen contains a variety of constituents, particularly fructose, which can be utilized for metabolic energy. Sperm utilize glucose in preference to fructose (43), but either can be used as an exogenous energy source.

The detrimental effects of bacteria on sperm and subsequent fertility of extended semen were first overcome, in part, by the addition of sulfanilamide to the medium (31), and subsequently, by the inclusion of penicillin and streptomycin (2) to extenders. The combination of peni-

development of ambient temperature extenders in that country. The addition of caproic acid to the IVT-CUE type of extender, followed by gassing with nitrogen (100), resulted in a new extender called "Caprogen." Caprogen successfully maintained maximum fertility for a 1-day period at ambient temperature which was sufficient for the needs of New Zealand breeders.

Another extender, designed to reduce the amount of perishable egg yolk in the ambient temperature medium, contains 15% coconut milk as a nutrient source (CME extender). For ambient temperature use of this latter extender, as little as 0.5% egg yolk may be used (103). CME extension of semen results in excellent fertility for 4 or more days (104), but similar extenders without coconut milk and with low egg yolk levels maintained similar fertility (86).

IVT has been used for preserving boar semen at temperatures above 5°C (43) and CME is effective in preserving buffalo sperm, but little work has been done with ambient temperature preservation of semen from other species. Lapwood and Martin (78) used saline-sugar extenders containing veronal-HCl, sodium sulfate or sodium tetraborate, with or without the addition of milk solids, to maintain ram sperm at body temperatures.

## 2. Preservation of Semen at 5°C

Most semen used for inseminations prior to the availability of frozen semen was maintained at refrigerator temperatures (4°C–5°C) to reduce metabolic rates and prolong sperm fertility. The first extenders in use were the yolk-phosphate-based media, but improved microscopic visibility of sperm in yolk-citrate extenders (44) led to widespread use of the latter. Many modifications have been made to the basic citrate extender (141), but it remains the most widely used formula for sperm preservation, with 20% egg yolk in buffer the generally accepted level for protection against cold shock (45).

Heated homogenized or skim milk has been used as an extender for semen from bulls (3), boars (101), horses (93), and dogs (62), either alone or in combination with sugars (141), glycerol (175), or egg yolk (89).

## 3. FROZEN SEMEN

Although earlier observations suggested that sperm cells could survive freezing, the real idea of storing frozen semen was not accepted until the serendipitous discovery that glycerol would protect the cells from freeze

damage (118). Since that time, freeze preservation has been developed and used for bull sperm and is being developed for most other species.

## 1. Cryoprotective Agents

The addition of glycerol to 5–10% concentrations in semen extenders is routinely used for freeze preservation of bull sperm, with 7% most common for yolk-citrate and 10% used with milk extenders (41, 141). These levels of glycerol, however, tend to be toxic to sperm from some other species, so lower (1–2%) concentrations are frequently used for freezing sperm from stallions and boars (49).

A number of other agents have been investigated for cryoprotective properties, including ethylene and propylene glycol (20, 150), dimethyl sulfoxide (20, 32, 108, 109, 152), high levels of nonpenetrating sugars such as lactose, raffinose, and arabinase (15, 16, 98, 139), and high levels of metabolizable sugars, such as fructose (28). The best cryoprotective results have been found, in general, when combinations of these chemicals are used, often to reduce the amount of glycerol necessary for cryoprotection (139).

## 2. Packaging Frozen Semen

Sperm are commonly packaged in one of three ways: (a) glass ampules, normally containing 0.5–1.2 ml of frozen semen; (b) polyvinyl chloride straws with a volume of 0.25 to 0.5 ml; (c) pellets containing about 0.1 ml. In some cases, larger ampules or straws (22, 75, 114), gelatin capsules (38), or polyvinyl plastic bags (35, 83) have been used to freeze larger volumes of semen.

Since the use of frozen semen was developed for the cattle industry, 1.0-ml glass ampules were used almost exclusively for storing the sperm. This method of packaging permitted automated labeling, filling, and sealing of sperm in packages suitable for a single insemination. As reviewed by Pickett and Berndtson (114), however, reports of superior sperm livability, greater storage efficiency, and higher conception rates resulting from sperm stored in plastic straws have led to rapid increases in the use of this packaging technique. Although the 0.25-ml straw has been popular in Europe (24), the greater adaptability of 0.5-ml straws to automated handling has led to their preferential use in the United States (114, 85). Diluted semen is held in straws by polyvinyl chloride plugs, nylon plugs, steel balls, or electrostatic compression.

Nagase and his colleagues in Japan (96–98) developed a method of pelleting semen for frozen storage by dropping about 0.1-ml drops of ex-



TABLE III

The Influence of Amylase and  $\beta$ -Glucuronidase on the Fertility of Frozen Bull Sperm\*

Enzyme	Level ( $\mu$ g/ml)	Percentage of control
None	—	100
$\alpha$ -Amylase	1	102
	10	104
$\beta$ -Amylase	1	104
	10	103
$\beta$ -Glucuronidase	150	106

\* Values are unweighted means calculated from the data of Hafs *et al.* (56, 57, 72), Sullivan and Elliot (161), and Linford (80).

would improve fertility of bull sperm. Subsequently, Hafs *et al.* (57) reported that  $\beta$ -glucuronidase increased bovine fertility beyond that obtained with amylase. These workers (56) also showed that  $\alpha$ -amylase was as effective as the  $\beta$  form in improving fertility. Other laboratories have confirmed these findings (80, 161; Table III) and have suggested that the effects of the enzymes might be additive (80).

Studies have been conducted on the addition of hyaluronidase (90) on fertility, but no consistent beneficial effects were found. The addition of steroid hormones such as androstenedione, estrogen, progesterone, and testosterone at high levels (40  $\mu$ g/ml), seems to depress respiration in rooster (146), bull (8), and ram (95) semen, but no effects of estrogen or progesterone were found in fertility trials (90, 157). Similar results were found when prostaglandins (154) or other hormones (90) were added to semen.

Altered respiration has been found following the addition to sperm of follicular fluid (51, 105), oviduct or uterine fluids (64, 105), cyclic AMP (79), caffeine (65), or a variety of other chemicals, enzymes, and hormones (90); none of these agents has been shown to have an effect on fertility of the semen.

## B. ALTERING SEX RATIO

Experiments designed to separate sperm bearing a Y chromosome (male-producing) from those containing an X chromosome (female-producing) have been conducted for more than 40 years. Attempts have

involved the use of treatments designed to selectively promote or destroy one type of sperm or to physically separate one from the other.

Procedures based on a variety of theories have been attempted including early insemination or acidic condition to increase female offspring (9, 61), antisera to differentially neutralize sperm (14), separation by sedimentation (27), electrophoresis (55), centrifugation (69), altered atmospheric pressure (68), and use of media of varying pH (29), but none of these procedures has been highly successful (69, 143). Sperm fractions collected after treatment must be tested by inseminating females and determining the sex of the resulting offspring. The process requires extended periods of time and large numbers of animals.

A major advance in distinguishing Y-bearing human sperm was reported in 1970 (10) when a quinacrine-staining body was identified as the Y chromosome but conclusive verification is lacking. Attempts to use this technique in nonprimates have not been successful. Eriessson *et al.* (39) reported that quinacrine-staining sperm were more highly motile than nonstaining sperm and that a preparation of almost entirely "Y-bearing" sperm could be separated from the mixture of X- and Y-bearing cells (leaving behind a mixture of X-bearing and slower-moving Y-bearing sperm). This enrichment of one sperm type has been repeated in one laboratory (155) and the concept of unequal motility has been supported in others (67, 129), but contradictory results exist (40, 130).

As reviewed by Schanbacher (143), Bhattacharya (unpublished) claims to have modified the quinacrine technique for use in bovine sperm but convincing data on sex of offspring with such sperm are not yet available.

A possible "spin-off" benefit of studies designed to increase the proportion of sperm of one type was noted in the studies with human sperm (39, 130, 155). In each case, the treatment increased the proportion of morphologically normal, highly motile sperm, whether separation occurred or not. Electrophoresis of bull sperm did not separate X- and Y-bearing cells, but increased fertility by 10%, compared with control sperm (55).

Although most treatments which influence the testis are without effect on sex ratio (42), recent work with the Chinese hamster suggests that treatment of males with caffeine (172) or theophylline (173) selectively reduces the development or viability of Y-bearing sperm, resulting in a proportional increase in female offspring.

### C. HETEROSPERMIC INSEMINATION

As discussed in Section III,C, the mixing of semen from different males frequently results in a ratio of offspring different from 1:1, even if equal

numbers of viable sperm are used (11, 115). Furthermore, the rate of fertility following heterospermic insemination is generally superior to the mean rate following homospermic insemination with the same males (12, 63, 115, 158). Since improved fertility could be expected on the grounds that eggs would automatically tend to be penetrated by the more fertile semen component in a mixture, one might expect fertility of a heterospermic mixture to approach (or equal) that of the best component in the mixture (158). If two or more males are considered equal in other ways, if differences in fertility have not been established, and if pedigree of the offspring is not of importance (or can be established by characteristics of the offspring), heterospermic insemination may be utilized to improve overall fertility of semen samples.

## VI. Insemination of the Female

A complete listing of techniques for the insemination of domestic animals is beyond the scope of this chapter; excellent descriptions of these procedures are available (52, 53, 88, 110). Maximal fertility will result if viable, healthy sperm are deposited in sufficient numbers at the proper site in the female tract at the optimum time in relation to ovulation. Skillful herdsmen, technicians, and proper semen handling are all requisite to this end.

### A. DETECTION OF ESTRUS

Since the fertile life of eggs in most species is relatively short and sperm may require capacitation before they are capable of fertilizing ova (Chapter 11), insemination should precede ovulation. Ovulation is difficult to determine routinely, so inseminations are usually related to the time of onset of estrus.

Estrus in the cow is characterized by the psychic manifestations of heat. The cow may bawl frequently and is usually restless. She may attempt to mount other animals and will stand to be mounted. The vulva is swollen and mucus is often secreted. Similar, but generally less pronounced, indications of estrus are found in ewes, sows, and mares.

Estrus can be recognized by frequent, careful observation of animals, especially when reproductive records are available to help determine the expected time of estrus. Where animals cannot be observed by the trained herdsmen, detection can be improved with the use of vasectomized males with chin ball (bulls) or harness (rams) markers (77, 88), testosterone-

treated females (73), or pressure-sensitive indicators on the rump of females (147). In some cases, inseminations are conducted at an appointed hour following treatments designed to synchronize estrus, whether symptoms of heat are observed or not (33, 122, 144, 153, 165).

## B. TIME OF INSEMINATION

Early work by Trimberger (166) and Trimberger and Davis (167) showed that maximum fertility was achieved in cows inseminated from midestrus to the end of estrus (Fig. 6). Since then, others have studied the period of fertility in detail, and confirmed the results of these workers.

Few data are available on the optimum time of insemination in other species, but indications are that the ewe should be inseminated about 11

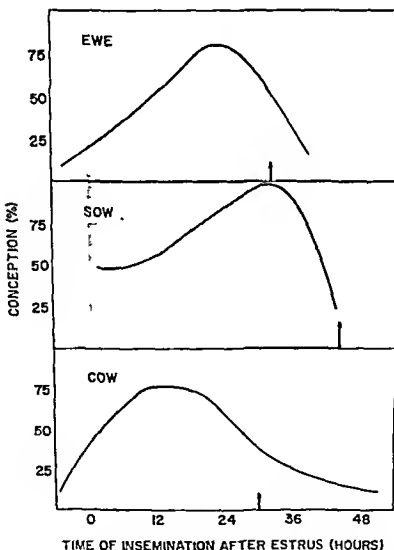


FIG. 6 Fertility of ewes, sows, and cows following insemination at varying times in relation to the onset of estrus. The shaded areas designate the duration of estrus. The arrow represents the time of ovulation.

to 15 hours before ovulation (33) or 16–24 hours after the onset of estrus (17, 113) for maximum fertility (Fig. 6). Sows inseminated between 9 and 15 hours before ovulation (33), corresponding to 30–36 hours after the beginning of estrus (Fig. 6), also exhibited maximum fertility (122).

Mares usually ovulate 24–48 hours before the end of estrus, which ranges in length from 2 to 7 days. If previous records on length of estrus are not available, the mare is first inseminated on the third or fourth day of estrus, then on alternate days for the duration of standing estrus (37, 113).

### C. SPERM NUMBERS AND INSEMINATION VOLUME

As shown in Table II, the numbers of sperm required for an insemination vary widely between species. This is due not only to inherent differences in animals, but also partially reflects the "state of the art" in collecting, preserving, and depositing sperm cells for maximum fertility. The commonly accepted value for a minimum number of motile cells necessary in bovine inseminations is ten million (Table II), requiring the freezing of up to 30 million sperm in each ampule. The use of straws results in better recovery of motile cells and reduced retention of cells in the storage package and insemination rod, thereby reducing the number of total cells needed for freezing and the number of motile cells needed for insemination (to 3–6 million; 114). Improved extenders and use of semen without freezing have allowed researchers to obtain satisfactory fertility with as few as 2.5 million total sperm per insemination (148).

The values shown in Table II for species other than the cow approach the lower limits with which satisfactory fertility has been obtained or reflect the numbers of cells routinely used in those species where widely varying cell numbers have not been used.

When similar numbers of total living cells are used, insemination volume is relatively less important except in swine. Volumes of 75–100 ml of extended semen are routinely used for inseminating sows and gilts (33, 88, 110, 122) and 50 ml appear minimal. For many years, larger volumes of semen (10–100 ml) seemed essential for maximal fertility in mares, but recent data have shown that a dose as low as 0.6–1.5 ml may be sufficient (113).

Although 1-ml doses of semen have resulted in satisfactory fertility in cattle for many years, the use of smaller straws generally increases conception rates (114), probably because of more uniform freezing and thawing rates; indeed, 0.25-ml straws may be superior to 0.5-ml straws in this respect (24).

## D INSEMINATION TECHNIQUE

In the cow, the best fertility can be obtained with the rectovaginal technique (160), with deposition of semen in the anterior portion of the cervix or in the uterine body (82) for first inseminations. Subsequent inseminations should be in the anterior cervix only, to prevent disruption of existing pregnancies (44 141). Use of vaginal inseminations or speculum-assisted inseminations gives poorer results in cattle (160).

In animals too small for use with the rectovaginal technique, use of a speculum (sheep and goat), guiding the insemination rod into the cervix with a gloved finger (dog and horse), or introduction of the inseminating rod without use of intravaginal guidance (pig, cat and rabbit) are alternatives. Where possible semen should be deposited into the uterus, in some cases (sheep and dog), elevating the hind quarters of the female during or after insemination may be helpful (88).

Recent work by Piekett *et al* (113) indicates that excessive palpation of the reproductive tract before insemination may be detrimental to fertility of mares. On the other hand, stimulus of the reproductive tract, including massage of the clitoris after insemination (125), may improve conception rates in cattle.

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# 11 Fertilization, Early Development, and Embryo Transfer

Gary B. Anderson

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Much of what is known about fertilization and development of the early embryo has been learned from invertebrates and lower vertebrates. Greater availability and relative size of eggs from these nonmammalian species facilitated early studies of these developmental processes. Refinements in superovulation techniques, *in vitro* embryo culture systems, and equipment for micromanipulation have only recently allowed extensive study of the same processes in mammalian embryos. For reasons of econ-

omy and convenience, much of the research on mammalian embryos has been done using laboratory animals, especially the mouse and rabbit; hypotheses concerning fertilization and embryo development in domestic animals must rely heavily upon results obtained from other species. Current evidence suggests, however, that early development is remarkably similar among mammals and cautious extrapolation from laboratory to domestic mammals is possible.

In most mammals the ovulated oocyte is similar in morphology and size, approximately 100  $\mu\text{m}$  in diameter. Fertilized eggs spend a comparable period of time in the oviduct undergoing a series of similar mitotic divisions. Current information suggests that the embryos from different mammals have similar nutrient requirements and use many of the same metabolic pathways. It is with this knowledge that generalizations of fertilization and preimplantation development in domestic animals have been made. When available, information pertaining to domestic animals is given.

## I. The Egg at Ovulation

### A. MORPHOLOGY

The cytoplasm of a freshly ovulated oocyte is typically surrounded by the vitelline membrane, the zona pellucida, and the cumulus oophorus. The zona pellucida is a noncellular mucoprotein structure that immediately surrounds the vitelline membrane. It is formed during follicular development by gradual accumulation of material between the oocyte and follicle cells. The zona pellucida is penetrated by egg and granulosa cell microvilli which may provide biochemical communication between the oocyte and the follicle (82). It probably functions both as mechanical protection for the egg and by regulating the immediate chemical environment of the egg (82). Survival of early embryos is reduced when the zona pellucida is damaged or removed (29, 74, 98). In some species it also provides a barrier to fertilization by more than one sperm cell and may prevent embryos from fusing during development.

The cumulus oophorus consists of layers of loosely packed granulosa cells. The granulosa cells closest to the zona pellucida are tightly packed into several layers, the corona radiata. A viscous matrix thought to be high in hyaluronic acid holds the granulosa cells of the cumulus oophorus together. Cells of the cumulus oophorus cling to the egg for several to many hours following ovulation and may play some role in fertilization. The cumulus oophorus is lost rapidly from eggs of the sheep, cow, and sow following ovulation (82).

## B. STAGE OF MATURATION

Meiosis is initiated in the fetal ovary, but is interrupted at the diplotene stage during prophase I (35). In most mammals, meiosis I is resumed following the ovulatory surge of gonadotropin. The chromatin then condenses, the germinal vesicle breaks down, and the first polar body is extruded. Meiosis II is halted at metaphase. It is at this stage, metaphase II, that the oocytes of most mammals are ovulated. Notable exceptions include eggs of the horse, dog, and fox which are thought to be ovulated prior to completion of meiosis I and where sperm penetration of the primary oocyte can occur (35). The stimulus for induction of maturation in these species is unknown.

When full-sized oocytes from the cow, sheep, pig, rabbit, mouse, rat, hamster, guinea pig, monkey, and man and possibly other species are removed from the follicle and placed in suitable culture medium, they spontaneously resume meiosis and proceed to metaphase II. Inhibition by some component of the follicle or follicular fluid which prevents oocyte maturation has been theorized. Although oocytes matured *in vitro* have low fertilizability, *in vitro* matured mouse oocytes have been shown to be capable of undergoing fertilization and developing into fetuses when transferred to foster mothers (33).

## II. Gamete and Embryo Transport

Ovulation results in the release of one to several eggs by rupture of the Graafian follicle(s). In domestic mammals survival time of gametes is short and deposition of spermatozoa in the female tract must be closely synchronized temporally with ovulation in order for normal fertilization to occur. Freshly ovulated oocytes are picked up by the fimbria of the infundibulum and directed into the oviduct. Sperm cells are usually ejaculated into the vagina (cow, sheep, rabbit, and man) or uterus (horse, dog, pig, and rodents). Since fertilization occurs in the ampulla of the oviduct, synchronous transport of oocytes down the female tract to the site of fertilization must be coincident with transport of sperm cells from the site of deposition. The female reproductive tract employs carefully controlled mechanisms whereby eggs and spermatozoa are transported in opposite directions during a limited period of time.

### A. TRANSPORT OF THE OOCYTE TO THE SITE OF FERTILIZATION

The mechanism by which freshly ovulated eggs are transported from the ruptured follicle into the oviduct depends upon a number of factors.

Included in these are the anatomic configuration of the fimbria and its relationship to the surface of the ovary at the time of ovulation, the manner by which the egg and surrounding granulosa cells are expressed from the follicle, and the physical characteristics of the antral fluids and matrix of the cumulus oophorus (20). At ovulation in the rabbit, guinea pig, cat, ungulates, and primates, the fimbriae become engorged with blood and contractile, and surround the ovaries. The freshly ovulated oocytes in the associated cumulus mass come into immediate contact with the ciliated cells lining the fimbriae which help to direct the oocytes into the oviduct. In the mouse, rat, and hamster the ovary is enclosed completely by a thin-membraned periovarial sac into which a relatively small fimbria projects. The fimbria makes only superficial contact with the ovaries and eggs are shed into the fluid-filled periovarial sac. Contractions of the mesovarium result in movement of the ovary, fluid, and eggs within the periovarial sac. When the eggs come into contact with the fimbria they are swept into the oviduct by ciliated cells.

Eggs are usually transported rapidly from the infundibulum to the site of fertilization above the isthmoampullar junction. Transport time has been estimated to be between 30 and 180 minutes (38) with as little as 6 minutes reported in the rabbit (22). Transport through the first part of the ampulla in the rat and rabbit is thought to be effected primarily by the action of cilia. Transport through the remainder of the ampulla is primarily by peristaltic and segmental muscular contractions, although cilia are present throughout the ampulla. Cilia may provide the primary means for transport through the entire ampulla in the cat and monkey (20). Movement of fluid through the ampulla may also provide a means of oocyte transport in some species. In polytocous animals the mass of cumulus cells and ova are often transported as a single entity. After fertilization embryos remain above the isthmoampullar junction for a variable number of hours depending upon the species (20).

## B. TRANSPORT OF THE EMBRYO THROUGH THE OVIDUCT

In most mammals, transport of ova through the oviduct to the uterus takes 3-4 days (Table 1). While rabbit ova reach the junction of the ampulla and isthmus as quickly as 6 minutes after ovulation, more than 50% are still above this junction 24 hours later. The remainder of the time in the oviduct is spent in slow transport through the isthmus (21). Mouse ova are transported rapidly through the ampulla and then detained at the isthmoampullar junction for 24 hours. Transport through the isthmus is again rapid with a 30-hour delay at the uterotubal junction (38).

In the pig (20, 80) and cow (2), ova spend most of the time in the

**TABLE I**  
**Rates of Development of Various Species<sup>a, b</sup>**

Species	One-cell stage (hours)	Morula stage (hours)	Blastocyst stage (days)	Entry into uterus (days)	Gestation (days)
Mouse	0-24	68-80	3-4	3-4	21
Rat	0-24	72-80	3-4	3-4	22
Rabbit	0-14	48-68	3-4	3-4	30
Cat	—	—	5-6	4-8	60
Pig	0-15	72-96	5-6	2-4	115
Goat	0-30	120-140	6	4	147
Sheep	0-38	96	6-7	2-4	150
Man	0-24	96	5-8	3	270
Cow	0-27	144	9	3-4	284
Horse	0-24	98	6	4-5	340

<sup>a</sup> Adapted from Brinster (28), McLaren (69), and Davies and Hesseldahl (34)

<sup>b</sup> Times estimated from ovulation.

oviduct above the isthmoampullar junction and then are transported rapidly through the isthmus into the uterus.

It is not known whether the isthmoampullar junction acts to retain embryos in the ampulla in all mammalian species. It is also not known how spermatozoa are allowed to enter the ampulla and effect fertilization in species where further egg transport is inhibited. In some species low doses of exogenous estrogen cause a phenomenon known as "tube locking" where ova are retained at the isthmoampullar junction; larger doses of estrogen hasten transport through the isthmus and into the uterus (38). It has been suggested that both estrogen and progesterone play a role in embryo transport.

Fertilization usually occurs with the cumulus mass still intact, but the egg is denuded by the time it enters the isthmus. Transport through the isthmus appears to be a complex process related to a pattern of segmental peristaltic and antiperistaltic contractions that have the capacity of moving the embryos backward and forward in a rotary fashion. In the rabbit, opossum, dog, and horse, a mucopolysaccharide coat, or mucin coat, is deposited outside the zona pellucida while the egg is in the isthmus. On approximately the fourth day following ovulation, the embryo passes from the isthmus through the uterotubal junction into the uterus.

### C. TRANSPORT OF SPERM TO THE SITE OF FERTILIZATION

In most mammals, spermatozoa are deposited in the vagina, cervix, or uterus of the female and must be transported to the ampulla of the oviduct



in order for fertilization to occur. Some authors have ascribed a role to sperm motility in normal transport while others consider sperm transport a passive process, totally a function of the female tract (47). Many physiological factors, including the volume of ejaculate, site of deposition, and anatomy of the female tract affect the rate of transport. Table II describes some of these variations. It should be noted that these values were obtained by a number of different workers using a variety of techniques and, therefore, variations in the estimates exist. It can be noted, however, that regardless of species, sperm transport through the female tract is fairly rapid with a relatively few sperm cells ultimately reaching the site of fertilization. In the rat, mouse, and rabbit, fewer than 500 of the estimated 50-500 million sperm cells ejaculated reach the ampulla of the oviduct (38).

In the horse and pig, a large volume of semen is ejaculated and at least part of that volume is propelled through the cervix into the uterus. In rodents, a small volume is ejaculated, but ejaculation is directly into the uterus. In these cases, the cervix of the female provides no barrier for transport of spermatozoa. In other animals, such as the cow, ewe, rabbit, and human female, sperm cells are deposited into the vagina and must be transported through the cervix before reaching the uterus and oviduct. In

**TABLE II**  
**Characteristics of the Ejaculate and Sperm Transport in Various Species\***

Species	Volume of ejaculate (ml)	Site of deposition	Interval from ejaculation to appearance of sperm in oviducts	Number of sperm reaching site of fertilization
Mouse	>0.1	Uterus	15 minutes	17+
Hamster	>0.1	Uterus	2-60 minutes	Few
Rat	0.1	Uterus	15-30 minutes	5-100
Guinea pig	0.15	Uterus	15 minutes	25-50
Rabbit	1.0	Vagina	3-6 hours	250-500
Cat	0.1-0.3	Vagina and cervix	No data	40-120
Dog	10.0		Several minutes to several hours	5-100
Sheep	1.0	Vagina	Several minutes to several hours	600-5,000
Cow	4.0	Vagina	2-13 minutes	4,200-27,500
Pig	250	Cervix and uterus	30 minutes	Few
Man	3.5	Vagina	30 minutes	Few

\* Adapted from Blandau (20)

these animals the role of a sperm reservoir has been suggested for the cervix; sperm migrate into the cervix and are slowly released into the uterus over a period of hours. By virtue of this constant flow of sperm from the cervix, and phagocytosis of sperm within the tract, it is theorized that a population of viable sperm can be maintained in the oviducts, particularly at the site of fertilization (78).

The way in which sperm cells are transported through the cervix is still subject to debate. One theory proposed to explain the rapid transport through the cervix is the "in-suck" theory (20). This theory states that contractions of the vagina and uterus during intromission and orgasm in the female result in sucking of seminal fluid into the cervical canal. This theory has been used to explain sperm transport through the cervix of the human female, but it is still a matter of controversy.

Seminal plasma in some species is capable of penetrating cervical mucus *in vitro* and facilitating the migration of sperm across a glass slide coated with cervical mucus. Sperm cells can also penetrate cervical mucus. These findings have been interpreted as possible means for facilitated transport of spermatozoa across the cervix by invasion of cervical mucus by seminal plasma and motile sperm (20, 38, 73). Passage of sperm through the cervix in the rabbit is enhanced by a second coital stimulus with a vasectomized male (13), suggesting involvement of either a neurohumoral response or a substance within the seminal plasma that stimulates muscular activity.

Transport of spermatozoa through the uterus to the uterotubal junction is rapid and is primarily the function of uterine muscle contractions. Oxytocin released during mating in the cow and sheep increases uterine activity and may facilitate sperm transport. Prostaglandins in the semen may have a similar effect.

Transport of spermatozoa through the uterotubal junction is complex because of the differences in anatomy and histology observed in different species (53). It has been suggested that the uterotubal junction selects against dead sperm and sperm of a foreign species. While such selectivity may exist in the rat, it is not seen in the sow (20, 45).

Transport of sperm through the isthmus in mammals is also accomplished primarily by muscular contractions. In the pigeon and painted tortoise, two types of cilia exist which beat in opposite directions and result in transport of sperm up the female tract and the eggs down (81). In many mammals, however, cilia beat toward the uterus so sperm transport in the oviduct is controlled by muscular contractions. A mechanism involving both cilia and muscular contractions has been recently described in the sow (20a).

### III. Fertilization

#### A. SPERM CAPACITATION

In 1951, Austin (3) and Chang (30) independently reported that freshly ejaculated rat and rabbit spermatozoa are incapable of penetrating an egg. After spending a period of time in the female tract they acquired fertilizing ability, a process termed capacitation. The original observations have been extended to include sperm of the rabbit, rat, hamster, ferret, sheep, pig (5), cat (55), mouse (61), guinea pig (117), and possibly the cow (64), monkey, and man (5, 37, 41). While the need for incubation of spermatozoa in the female tract prior to fertilization has been established, the time required varies with the species. Mouse spermatozoa require less than 1 hour while rabbit sperm require at least 5-6 hours to attain full fertilizing potential. Sheep and pig sperm require 1½ and 3-6 hours, respectively (6). Sperm from some species where capacitation has not been shown to be necessary may undergo the process so rapidly that it escapes detection.

Sperm capacitation has been viewed as composed to two mechanisms, removal of a chemical factor (decapacitation factor) from the sperm cell and activation of acrosomal enzymes (acrosome reaction).

Chang (31) observed that capacitated rabbit spermatozoa lose their ability to fertilize when incubated in rabbit, bull, or human seminal plasma. These "decapacitated" sperm regained their fertilizing ability after further exposure to the female tract. These observations were interpreted to mean that a decapacitation factor exists in the seminal plasma which coats the sperm cell and must be removed before the sperm gain fertilizing ability. Decapacitation factor has been found in fluids throughout the male tract including the testis and epididymis, but the role it plays in capacitation is still debated. Corona-penetrating enzyme, present in the acrosome of the sperm cell and thought to be necessary for penetration of the corona radiata, is inactivated by decapacitation factor (107). Removal of the decapacitation factor might then result in the activation of one or more acrosomal enzymes, including acrosin and neuraminidase-like enzyme, necessary for fertilization (56). Capacitated sperm bind to the zona pellucida of the egg better than decapacitated or uncapacitated sperm suggesting that removal of the decapacitation factor results in a membrane alteration. It has also been suggested that decapacitation factor binds to the sperm cell membrane and stabilizes it against premature release of acrosomal enzymes (12). Whatever the mechanism, capacitation includes the removal or inactivation of seminal plasma antifertility agents bound to the sperm head.

A membrane phenomenon called the "acrosome reaction" has also been shown to occur prior to fertilization in some species. Some workers con-

sider the acrosome reaction as part of the capacitation process (107) while others consider it a process that occurs subsequent to capacitation (15). Regardless of the interpretation, it is thought to be essential for fertilization and has been described in spermatozoa of several laboratory species and man (89a); its importance in fertilization in domestic livestock is not established (8, 12).

The acrosome reaction involves the progressive breakdown and fusion of the plasma membrane and outer acrosomal membrane of the sperm cell (70) (Fig. 1). Vesicle formation allows leakage of enzymes from the acrosome with ultimate loss of the plasma membrane/acrosome complex leaving the inner acrosomal membrane exposed. This release of acrosomal enzymes may be particularly important in the penetration of the cumulus oophorus and corona radiata. In general, it is believed that capacitated sperm begin to undergo the acrosome reaction, with consequent liberation of enzymes, in close proximity to or at initial contact with the cumulus/egg mass. Initially, enzyme is probably released gradually to dissolve the matrix between the granulosa cells thereby facilitating passage of the sperm toward the zona pellucida. Complete loss of the outer acrosome membrane occurs before the sperm cell penetrates the zona. In fact, some authors have theorized that loss of the acrosome exposes a "perforatorium" which acts as a mechanical tool to help the sperm penetrate the zona (120). Despite the tentative nature of existing information regarding the acrosome reaction it is clear that it represents a morphological change that occurs in the female tract of some species and is important in subsequent fertilization. The role of the cumulus cells in inducing the acrosome reaction and their role in other mammalian species remain subjects to debate.

Capacitation occurs in both the uterus and oviduct, although sperm are more readily capacitated if exposed to both uterine and oviductal environments. Most work dealing with the hormonal control of capacitation has been done using the rabbit as a model. In general, estrogens have a stimulating effect upon capacitation while progesterone administration can inhibit capacitation. These observations are in line with expected results since mating and the need for capacitation normally occur when the female is under the influence of high levels of estrogen and is receptive to the male. Capacitation in the uterus seems to be more responsive to hormones than capacitation in the oviduct.

## B. SPERM PENETRATION

In most mammals, the sperm cell that successfully fertilizes the egg must be capable of traversing the cumulus oophorus, corona radiata, and zona pellucida before it can cross the plasma or vitelline membrane of the egg.

Hyaluronidase is thought to be released during the acrosome reaction and is important in dissolving the hyaluronic acid complex matrix of the cumulus oophorus as the spermatozoan passes through. A second acrosomal enzyme, corona-penetrating enzyme, helps the sperm move between the cells of the corona radiata by dissolving the matrix that holds these cells together. One or a series of acrosomal proteolytic enzymes, including acrosin (also called trypsinlike enzyme or acrosomal proteinase) are thought to allow the sperm cell to digest its way through the mucoprotein zona pellucida (Fig. 1). The term "zona lysis" has also been assigned to the substance responsible for sperm penetration of the zona pellucida. It is thought that sperm motility is essential during this phase of fertilization since the whipping motion of the sperm tail facilitates passage through the outer investments of the egg.

Experimental evidence suggests that the zona pellucida exhibits a species-specific receptor. Therefore, sperm from foreign species are unable to attach and penetrate. If the zona pellucida is removed from rat or mouse eggs, sperm from hamsters or guinea pigs are still unable to penetrate the vitelline membrane. Zona-free hamster eggs, on the other hand, can be penetrated by capacitated guinea pig, mouse, and rat sperm indicating the specificity rests in the zona pellucida in this species (119).

Incorporation of the sperm cell by the cytoplasm of the egg has been described for the rat (83, 93), hamster (9, 120), and rabbit (11, 12). When the sperm cell comes in contact with the vitelline membrane, it lies flat on the vitelline surface with first contact established immediately posterior to the area previously covered by the acrosome. As soon as this contact between gametes has occurred, the membrane of the sperm fuses with the vitelline membrane of the egg. The egg cytoplasm around the area of contact elevates and actively surrounds the sperm head. The egg membranes fuse around the sperm head, incorporating it into the egg (122). The thick lamina resulting from the fusion of the two gamete membranes in the area of the original contact disappears resulting in direct exposure of the sperm nucleus to the egg cytoplasm (121). The sperm nuclear membrane disappears and releases the chromatin material which begins to disperse. During the early stages of this incorporation process the sperm cell, including the tail, is contained in the perivitelline space (122). Except in a few species such as the Chinese hamster and field vole, the entire length of the sperm tail is incorporated into the egg by progressive envelopment and fusion of the egg membrane over the sperm (120).

### C. CONSEQUENCES OF FERTILIZATION

Penetration of the egg by the sperm results in a series of phenomena leading to normal development of the fertilized egg. In most mammals, the

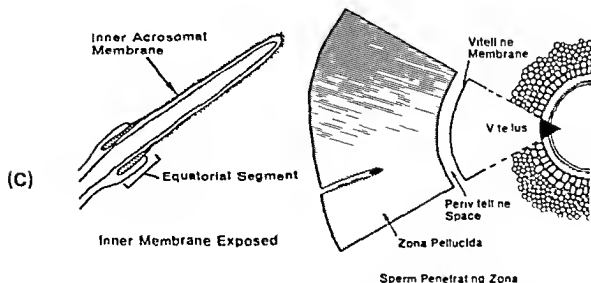
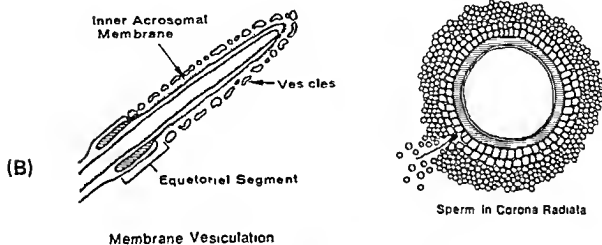
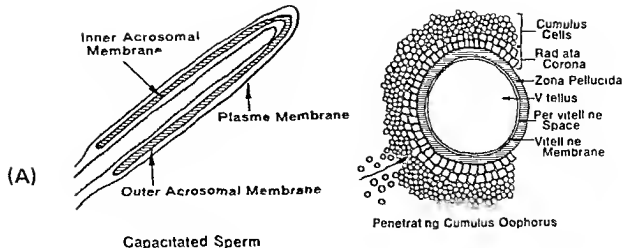


Fig. 1 Sperm acrosome reaction and penetration of outer investments of ovulated oocyte (A) Status of capacitated sperm (left) as it penetrates the cumulus oophorus (right) (B) Acrosome reaction of sperm (left) as it penetrates corona radiata (right) (C) Status of reacted sperm (left) as it penetrates zona pellucida. Reproduced from McRorie and Williams (70) with permission from *Ann. Rev. Biochem.* 43 Copyright © 1974 by *Ann. Rev. Inc.* All rights reserved

erection of a barrier to prevent fertilization by more than one sperm will occur. This barrier includes the zona reaction and the vitelline block. Penetration of the egg by the sperm also stimulates the resumption and completion of meiosis II with extrusion of the second polar body. The male and female pronuclei are formed and unite (syngamy) to establish the diploid, one-cell zygote. Fertilization also results in the activation of the egg to initiate cleavage, and enhanced biochemical activity.

## 1. Erection of a Barrier to Polyspermy

Polyspermic fertilization, when it does occur, often results in a polyploid individual which is not viable. It is therefore important that a mechanism exists to limit fertilization of the egg to only one sperm. The chances of polyspermy occurring are decreased by the limited number of sperm cells that reach the site of fertilization; deposition of excessive numbers of spermatozoa directly into the oviduct results in polyspermic fertilization in the pig (60). The egg also displays a mechanism for preventing penetration by more than one spermatozoan. The block to polyspermy in mammalian eggs operates at two levels in most species: the zona pellucida (zona reaction) and the vitelline membrane (vitelline block). The attachment of the fertilizing sperm cell to the vitelline membrane results in the disruption or breakdown of egg cytoplasmic structures called cortical granules. The membrane surrounding the cortical granules fuses with the egg plasma membrane and releases the contents of the granules into the perivitelline space (82). The release of the cortical granule contents results in some change in the character of the zona pellucida (zona reaction) which makes it impenetrable to other spermatozoa. At the level of the vitelline membrane, the contents of the cortical granules may coat the vitelline membrane and prevent attachment of other sperm cells or make the membrane unresponsive to sperm attachment (vitelline block) (82). Recent work with the mouse and hamster suggests that the cortical granules release a trypsin-like protease that attacks a species-specific "receptor-for-spermatozoa" on the zona pellucida (52). This theory is supported by observations in the hamster that sperm do not attach to the zona pellucida following sperm-induced rupture of the cortical granules (10). These same authors reported that the zona reaction developed in less than 15 minutes after cortical granule breakdown. Cortical granules have been identified not only in the hamster and mouse, but also in the rat, rabbit, and pig (4).

Most of the information we have regarding the barriers to polyspermy has been obtained from laboratory species. Work with other species has shown differences among the species studied. In some species (sheep, dog, and hamster) the zona reaction is relatively quick and effective and very

few sperm other than the one involved in fertilization are ever found in the perivitelline space. In the mouse and rat, extra sperm in the perivitelline space are more common which suggests that the zona reaction is slower in these species. The rabbit has no zona reaction at all and many sperm pass through the zona pellucida. However, an effective vitelline block allows only one spermatozoan to enter the egg cytoplasm and effect fertilization.

## 2. Completion of Meiosis II

At the time of sperm penetration in most domestic mammals, except the horse and dog, the egg is resting in metaphase of the second meiotic division. The chromosomes are arranged mostly in pairs and aligned on the equator of the meiotic spindle. Migration of the chromosomes to opposite poles of the spindle and cytokinesis result in the formation of the haploid second polar body and ovum. The second polar body can be distinguished from the first polar body by its smaller size and morphological features. The first polar body usually contains cortical granules as it is extruded prior to fertilization. In addition, chromosomes of the first polar body, like those of the egg entering meiosis II, contain no nuclear envelope. Those of the second polar body are enclosed, undergoing a process similar to the formation of the female pronucleus, at the end of meiosis II.

## 3. Pronucleus Formation, Syngamy, and Activation of Cleavage

The haploid number of chromosomes remaining in the fertilized ovum at the end of the second meiotic division becomes surrounded by a nuclear membrane. This structure is referred to as the female pronucleus.

Upon entry into the ooplasm, the sperm head begins to swell. The sperm tail and sperm head elements are separated from the nucleus and soon degenerate. The sperm nucleus continues to swell, acquires a nuclear membrane, and becomes the male pronucleus.

The male and female pronuclei move into close proximity to one another and may contact each other in some species (rabbit; 66), but not others (mouse; 122). The pronuclei lose their spherical shape and become irregular. There must be chromosome duplication at some point prior to chromatin condensation. As prophase of the first cleavage division is approached, nucleoli disappear from the pronuclei, the chromosomes condense and, finally, there is breakdown of the pronuclear envelopes. It has been observed in the mouse (122) and rabbit (66) that pronuclei do not fuse prior to pronuclear membrane breakdown and form a single nucleus as is common in some invertebrates. At the end of prophase, two distinct sets of



condensed chromosomes can be observed in the zygote. During metaphase the two groups of chromosomes move together and arrange themselves on the equatorial plate. As mitosis continues, the sister chromatids separate and migrate in opposite directions. With the formation of nuclear membranes and deepening of the cleavage furrow, two diploid blastomeres are formed marking the end of the first cleavage division.

#### D. AGING OF GAMETES

In most species, mating shortly precedes ovulation. The coincidence of these phenomena ensures that fertile sperm cells will be residing at the site of fertilization in the oviduct when the oocyte is released from the follicle. In species where sperm capacitation is necessary or prolonged, sperm must be in the female tract for several hours in order to fertilize the egg. Timing is very important since the fertile lives of the gametes are very short. Table III gives the approximate fertile lives of eggs and sperm from various species. Most mammalian eggs should be fertilized within a few hours of ovulation and few can be fertilized later than 12 hours after ovulation (a possible exception is the dog; 31a). In domestic animals the fertile life of sperm after ejaculation is also short. This fragility of the gametes

TABLE III  
Survival Time and Fertility of Gametes in the Female Tract\*

Species	Retention of sperm motility (hours)	Retention of fertility (hours)	
		Egg <sup>b</sup>	Sperm
Man	60-96	6-24	28-48
Rabbit	43-50	6-8	30-36
Cow	15-36	8-12	28-50
Sheep	48	16-24	30-48
Guinea pig	41	20	21-22
Mouse	13	6-15	6-12
Rat	17	8-12	14
Ferret	126	30	48-126
Horse	144	6-8	144
Pig	50	8-10	24-48

\* Adapted from Hamner (54a), McLaren (69), and Dukelow and Riegle (38).

<sup>b</sup> Values are approximate and based on estimated time from ovulation.

is particularly important to livestock producers utilizing artificial insemination. Accurate estrus detection is imperative to proper timing of insemination. Failure of fertilization to occur while the gametes are "fresh" can affect not only fertilization but also subsequent embryonic development. It has been shown that optimum fertility in the pig and sheep is achieved if insemination occurs 12 hours prior to ovulation (39). This procedure allows time for adequate numbers of capacitated sperm to reach the site of fertilization by the time of ovulation and prior to decreased fertility of the sperm cells.

Aging of eggs usually results in decreased fertilizability and an increase in abnormal embryos (87). Senescence appears to effect failure of the block to polyspermy and deterioration of the female genome and/or its division apparatus. In pig and hamster ova, aging is expressed as a deterioration of the egg's defenses against polyspermy, leading to penetration by more than one spermatozoan. The pig (87) also shows digyny in aged eggs, the formation of two female pronuclei resulting from retention of the second polar body or fragmentation of the female pronucleus. In aged mouse ova, on the other hand, fertilization occurs, but the female pronucleus fails to develop normally. If fertilization is delayed in the rabbit, eggs undergo a disruption of the meiotic apparatus evidenced by alterations in the orientation and structure of the meiotic spindle and wandering of chromosomes (65). Digyny and hypodiploidy have also been reported to occur in aged rabbit eggs (87).

Aging of the sperm cell in the female tract also has a detrimental effect upon fertility although probably little effect upon embryonic development. Table III gives the approximate intervals from ejaculation that spermatozoa remain motile and fertile in the female tract. The application of artificial insemination to domestic livestock has made the effects of *in vitro* aging of spermatozoa important. When sperm cells are held at 4°-5°C prior to insemination, fertility is decreased and embryonic mortality increases with increasing storage periods (87). Storage at -196°C delays, but does not prevent, gamete senescence. It has been suggested that these results are due to a time-dependent change in the genetic information contributed by spermatozoa.

## E. IN VITRO FERTILIZATION

The fertilization process can be accomplished *in vitro* in many species of mammals and has proved successful for the hamster, mouse, rabbit (23), rat (72), cat (55), guinea pig (118), pig (58), cow (40), mongolian gerbil (79), and human (41). Criteria for normal fertilization, however, have not in most cases included the production of viable young following

transfer to a suitable recipient female. With the exception of the pig and cow, *in vitro* fertilization techniques have not been successful in the live-stock species.

In most *in vitro* fertilization procedures, it is necessary to use sperm previously incubated and capacitated in the female tract. Several recent reports have indicated that capacitation can be effected *in vitro* in the golden hamster (116), mouse (61), guinea pig (118), and rat (96) allowing fertilization *in vitro* by epididymal sperm. Most *in vitro* fertilization procedures utilize recently ovulated oocytes or oocytes removed from follicles shortly before ovulation. In both cases extrusion of the first polar body has occurred. Only in the mouse has success been achieved with *in vitro* fertilization of oocytes removed from the follicle and allowed to complete meiosis I *in vitro* (33).

## F. PARTHENOGENESIS

Occasionally mammalian eggs become activated to develop without intervention of the fertilizing male gamete. This interesting process is called parthenogenesis, but it is only of academic interest at this time. For those interested in the subject, the following references are recommended (51, 94, 111).

# IV. Embryo Development

## A. CLEAVAGE

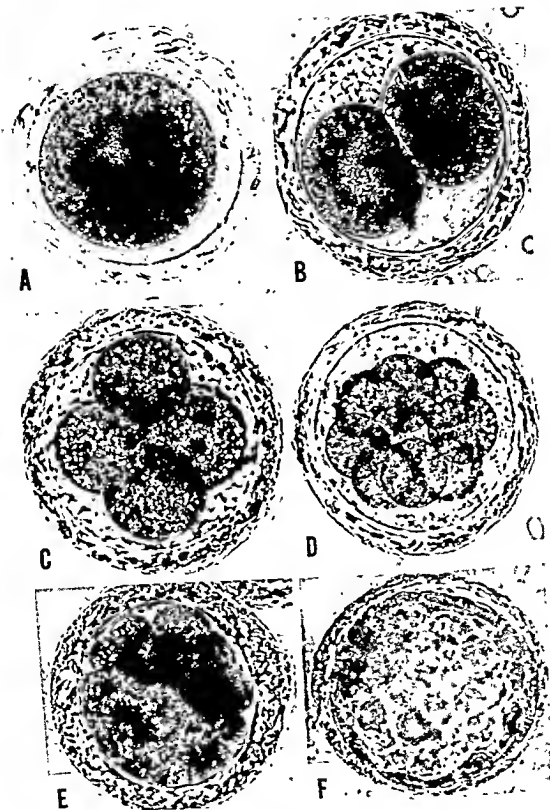
The fertilized mammalian egg begins its development as one of the largest cells of the body with a high ratio of cytoplasmic to nuclear volume. Following fertilization the ovum undergoes mitosis. The first mitotic division of the zygote results in the formation of a two-cell embryo. Each cell, or blastomere, is essentially the same size and is one-half the size of the original single cell. Unlike mitosis in other cells where the daughter cells go through a growth phase prior to the next division, both blastomeres of the two-cell embryo divide again yielding a four-cell embryo. Each blastomere is now one-quarter the size of the original single cell. The four blastomeres divide to form an eight-cell embryo, then a sixteen-cell embryo, and so on. Cell divisions are not completely synchronous and uneven numbers of blastomeres are seen. For example, it is not unusual to observe a three-cell embryo formed when one blastomere of a two-cell embryo divides slightly before the other. A normal three-cell embryo is easily distinguished by one large and two small blastomeres. Throughout these mitotic divisions,

cells become progressively smaller and smaller with no net increase in the size of the embryo. Essentially, the cytoplasm of the zygote is partitioned into smaller packets until a solid ball of cells called a morula is formed. The number of cells present at the morula stage is species dependent. Most species form a morula at approximately 16-32 cells. Some species, such as a rabbit, continue mitotic divisions, thus, the morula may contain a hundred cells. The size of the individual blastomeres of the morula is reduced by continued mitosis until it reaches that of normal adult body cells. This change in the cytoplasmic to nuclear volume ratio is thought to be important in the regulation of genetic action in the embryo (28). Not only does the mammalian embryo fail to increase in size during these cleavage divisions, but evidence in the mouse indicates that there is an actual decrease in total mass during the first several days of development (24). The morphology of the mammalian embryo during cleavage is illustrated in Fig. 2.

## B. BLASTOCYST FORMATION

Following the morula stage, the embryo undergoes cavitation resulting in the formation of a fluid-filled sphere, the blastocyst (see Fig. 2). The cavity is referred to as the blastocoele. The blastocyst stage marks the first overt sign of cellular differentiation in the developing embryo with the formation of at least two cell types. The outer single layer of cells is called the trophoblast and represents the cells from which the fetal chorion will develop. At one pole of the blastocyst is located a group of cells called the inner cell mass which will form the embryo proper. In the mouse, blastulation occurs at the 32-cell stage and only three or four of these cells form the inner cell mass whereas the remainder become trophoblast (48). It is not known whether this differentiation is due to cytoplasmic differences in the unfertilized egg which are reflected in the cells making up the blastocyst or if location of the cells in regard to one another is the primary determinant. According to the latter theory, cells which are forced to the interior of the morula differentiate into inner cell mass while the remainder become trophoblast.

The forces responsible for cavitation and accumulation of fluid between the cells of the morula are as yet undetermined. Fluid accumulation may be due to changes in permeability and active transport of selected ions in the trophoblast cells (34). Electrical potentials have been measured across the rabbit trophoblast and the inside has been shown to be negative to the outside. This potential difference is evidence for active transport across the trophoblast (32). Once the blastocoele is formed, blastocysts in many species go through a series of slow expansions followed by rapid contrac-



tions with ultimate increase in size. Rates of expansion and size changes are species-dependent. Blastocysts of the primate, rabbit, dog, cat, and most livestock species increase many times their size during the period of blastocyst expansion. In fact, the volume of the rabbit blastocyst increases 4000 fold between days 4 and 7 of development (28). Blastocysts of most laboratory rodents, including the mouse, rat, and guinea pig, show little blastocyst expansion, the total size of the implanting blastocyst is similar to that of the ovulated egg (54).

The formation of the blastocyst from a solid ball of cells prepares the embryo for gastrulation, the formation of the three primary germ layers (ectoderm, mesoderm, and endoderm) from which the various tissues and organs will develop (34). Expansion of the blastocyst may also function to "turn off" transport mechanisms of the uterus. When the blastocyst reaches a critical size, there may result an inhibition of propulsion which helps to orient the embryo in the uterus prior to implantation. Blastocyst expansion may also play a role in loss of the zona pellucida. Most mammalian embryos escape or "hatch" from the zona pellucida at some time prior to implantation. An exception may be the rabbit embryo which is thought to implant with the zona highly attenuated, but still intact. It is interesting to note that rabbit embryos cultured *in vitro* readily hatch from the zona. Expansion and the resulting thinning of the zona pellucida may play a role in the ultimate escape of the embryo. It has been suggested that blastocyst expansion acts in conjunction with an estrogen-sensitive uterine "zona lysis" to free the blastocyst from the zona pellucida (68).

## C RATE OF CLEAVAGE

Cleavage rates for embryos of a number of laboratory and domestic species are shown in Table I. The laboratory species generally have shorter gestation periods than domestic species and slightly greater rates of cleav-

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FIG 2 Pig embryos collected at various intervals from ovulation demonstrating development from fertilization to the blastocyst stage.  $\times 440$ . Sperm cells adhering to the zona pellucida are conspicuous. (A) One cell zygote. (B) Two cell embryo recovered from the oviduct 18 hours after ovulation. Note that each blastomere is one half the size of the original one cell zygote. (C) and (D) Four cell (recovered from oviduct) and eight-cell (recovered from upper uterine horn) embryos collected 41 and 55 hours after ovulation respectively. (E) Morula containing 16-32 cells recovered from upper uterine horn 73 hours after ovulation. Note that the size of the morula is the same as that of the one cell zygote. (F) Blastocyst recovered from upper uterine horn 117 hours after ovulation. The fluid filled blastocoele is evident. Expansion of the blastocyst has resulted in thinning of the zona pellucida. Reproduced from Hunter (60a).

age. As pointed out earlier, however, development during the first several days following fertilization is remarkably similar for most species studied. Passage from the oviduct into the uterus is coincidental with blastocyst formation in several laboratory species. This observation has led to speculation regarding the involvement of the uterus in initiating blastulation. Several workers have isolated endometrial products, including a protein from the rabbit uterus called blastokinin or uteroglobin (14, 63), which are thought to have stimulatory effects upon embryo development. Recent work, including the culture of embryos in chemically defined media, indicates that probably no single uterine factor controls development. The observation that embryos that develop *in vitro* often do so at a slower rate than those developing *in vivo* suggests, however, that the maternal environment *does* effect embryo development. Treatments that hasten or delay passage of the embryo from the oviduct to the uterus and asynchronous embryo transfers result in decreased embryo viability.

#### D. DIFFERENTIATION OF CELL TYPES

Cellular differentiation can be observed by the blastocyst stage with the formation and differentiation of trophoblast and inner cell mass cells. During the morula stage in some species, biochemical differences can identify prospective cells of these two types. There is evidence that single blastomeres of the early mammalian embryo are totipotent, or possess the potential to form a complete individual. When all blastomeres except one were destroyed in two-, four-, and eight-cell sheep and rabbit embryos, the remaining blastomere developed into a normal young following transfer to a suitable recipient (76). Single blastomeres of early cleavage stage pig embryos continue development for a time following destruction of the other blastomeres (75). Separation of the inner cell mass of 6- and 7-day sheep embryos into two parts has, in some cases, resulted in the birth of normal lambs following culture and transfer (97). Since the success rate in such experiments is usually low, it is impossible to be sure that each blastomere of an early cleavage stage embryo is totipotent.

The experimental production of allophenic individuals characterized by the presence of cells of different phenotypes in various tissues of the body also indicates early stage embryos have not differentiated. Allophenics can be produced by removing the zona pellucida and allowing embryos to fuse, or by injecting blastomeres from one embryo into the blastocoele of another. The resulting offspring possesses to varying extents the genotypes and phenotypes of the two original embryos. The production of allophenics has been described in the mouse (71), rat (67), and sheep (99).

## E. EMBRYO METABOLISM

The development of chemically defined media for the culture of mammalian embryos *in vitro* has provided a means for studying embryo metabolism during the preimplantation stages. To date, research in this area has been limited almost exclusively to the mouse and rabbit. Metabolism of the developing mammalian embryo has been the subject of a number of excellent reviews (17, 18, 25-28, 42, 100, 101) and will be discussed only briefly to emphasize the dynamic state of early embryo metabolism.

Energy requirements and carbohydrate metabolism have been studied during the preimplantation stages of the mouse and rabbit. These studies have shown that while the substrates utilized vary, both species show a gradual change in substrate requirements as development progresses. The mouse oocyte appears to require pyruvate for development; the two-cell stage embryo can also utilize lactate, oxaloacetate, or phosphoenolpyruvate. By the eight-cell stage the mouse embryo can utilize glucose as an energy substrate via the Embden-Meyerhof pathway. The rabbit embryo can utilize a number of energy substrates including glucose, pyruvate, lactate, and possibly amino acids during the early cleavage stages. The rabbit embryo oxidizes glucose via the pentose shunt up to the morula stage; the Embden-Meyerhof pathway and Krebs cycle predominate at the blastocyst stage. These changes may reflect differences in permeability or enzyme activity. The embryo can also store glucose in the form of glycogen.

Specific amino acid requirements have not been demonstrated for the cleavage stage mouse embryo. Protein synthesis occurs during this time, but it is not until the morula stage that synthesis exceeds degradation. Protein synthesis occurs also in the early stage rabbit embryo and increases greatly at the blastocyst stage. The rabbit embryo, unlike the mouse embryo, may have requirements for specific amino acids indicating they are not provided in sufficient quantities in the ovulated egg to support the protein synthesis that occurs.

The mouse embryo initiates synthesis of tRNA and rRNA at the four-cell stage. A similar picture is seen for tRNA in the rabbit, but rRNA is not synthesized until late cleavage. In the rabbit, protein synthesis during cleavage is presumably on polysomes whose ribosomes are of maternal origin.

These studies are of particular interest in providing information regarding activation of the embryonic genome. In invertebrate and amphibian species, the activity of the embryonic genome does not begin until gastrulation; everything required for development to this stage is provided by the egg. Some workers have suggested that gene activation does not occur



until the blastocyst stage in mammals (112). Other evidence has been summarized, including the expression of marker paternal alleles prior to the eight-cell stage of the mouse, suggesting in mammals that the embryonic genome is activated shortly after fertilization and affects early pre-implantation development (44).

## F. IN VITRO CULTURE

Much of what we know of the development of mammalian embryos has been gained from experiments using *in vitro* culture techniques. These procedures have allowed the scientist to monitor development under controlled environmental conditions. The use of chemically defined media rather than natural media, such as serum or oviduct fluid, has improved embryo culture systems. The variable composition of natural media makes the study of nutrient requirements impossible and increases variability of culture results. Techniques for culture from early cleavage to the blastocyst stage in defined media have been described for embryos of the mouse (19), rabbit (62), sheep (115), and cow (95). Embryos of these and other species have been cultured with varying success in natural media. Recent reports describe development of bovine embryos from the one- and eight-cell stage to the expanding and hatched blastocyst stage in media containing heat-treated fetal calf serum (113, 114) (Fig. 3). Mouse embryos have been cultured *in vitro* to the stage of a beating heart equivalent to 8.5 days of gestation (59).

Recent success has also been reported for preservation of mammalian embryos at both refrigeration and freezing temperatures. Such systems may find application in embryo transfer programs as well as provide a means for preserving strains of animals. Successful short-term storage at refrigeration temperatures has been reported for the mouse (102), rabbit (1), sheep (77), cow (92), and pig (108). Successful storage at  $-196^{\circ}\text{C}$  has been reported for the mouse (103, 104, 109), cow (110), sheep (106), rabbit (7), and rat (105).

## V. Embryo Transfer

Transfer of fertilized eggs from one female to another has been a useful research tool for a number of years whenever it was desirable to separate fetal and maternal genetic expression. Its use as a research tool has been prevalent in laboratory and domestic species. Recently, interest has increased in applying embryo transfer techniques to domestic animals, particularly the cow. Embryo transfer in livestock has been the subject of



FIG. 3 Development of bovine embryos *in vitro* (A) Eight cell embryos recovered 96 hours after estrus  $\times 375$  (B) Embryos in (A) after 140 hours in culture  $\times 375$  (C) Both blastocysts have begun the expansion process and one is 'hatching from the zona pellucida' (A) and (B) after 160 hours in culture  $\times 750$  (D) Embryo in (C) has completely escaped from the zona pellucida after 165 hours *in vitro*  $\times 375$  From Wright *et al.* (114)

several recent reviews (46, 49, 50). In North America, current application on a commercial scale is limited largely to efforts to rapidly upgrade to the exotic European beef breeds. However, research is being carried out in both North America and Europe to improve present techniques for other applications to domestic animals. Although there is some interest in applying embryo transfer techniques to other species, this discussion is limited to the cow.

Coupling superovulation techniques and embryo transfer may provide a means for increasing the number of offspring a genetically superior female is capable of producing. Such a procedure may hasten genetic gain through progeny testing and selection of females much as artificial insemination has done for the male. Embryo transfer may prove especially beneficial to livestock producers if embryos are collected while the female is young, even prepuberal, and responsive to superovulatory hormones. A female that is superovulated when 5-7 months of age can produce calves through embryo transfer before she reaches normal breeding age. Pregnancy rates following transfer of embryos from prepuberal calves have been low (88), but at the University of California, Davis campus, pregnancy has been achieved by transferring an embryo collected from a prepuberal calf. The donor, a 6-month-old Angus, was born from the transfer of an embryo collected from another prepuberal calf, a demonstration of the potential application to shortening the generation interval in livestock.

Induced twinning in beef cattle is another potential application of embryo transfer techniques. Experiments have indicated that twin pregnancy can be induced by transfer of two embryos to a recipient or transfer of one embryo to a previously mated recipient.

## A. TECHNIQUES AND PROCEDURES

Each commercial and research embryo transfer group has devised and modified procedures sufficiently that no single description of techniques is possible. However, some similarities in procedures exist among the different groups.

### 1. Superovulation

Superovulation can be induced with pregnant mare serum gonadotropin (PMSG) or a combination of FSH and LH. PMSG has the advantage that a single injection is sufficient to induce superovulation while FSH and LH must be given in a series of injections. The interval between gonadotropin injection and ovulation is critical. Initial injection of gonadotropin approximately 5 days prior to ovulation appears to induce the greatest

superovulation response. An injection can be given on day 16 of a cow's estrous cycle, a procedure that makes accurate estrus detection essential. Prostaglandin  $F_{2\alpha}$  or its analogs are being used experimentally in conjunction with gonadotropins to induce superovulation. Prostaglandin  $F_{2\alpha}$  induces luteolysis in the cow between days 5 and 17 of the estrous cycle. Administration of prostaglandin 2 days after PMSG stimulates fertile estrus 48–72 hours later (43, 57). Prostaglandin  $F_{2\alpha}$  and its analogs are not presently available for commercial use in the United States.

Administration of progesterone or a progestagen by implant, vaginal sponge, or daily injection may also be used in conjunction with gonadotropin treatment. Administration of a progestagen prevents estrus by inhibiting gonadotropin release; the cow shows estrus 48–96 hours after progesterone withdrawal. An effective treatment involves progesterone administration for 13 days with PMSG administration at progesterone withdrawal. A luteolytic dose of estrogen is given on the fifth day of treatment. Regardless of the method used, variability of response to the superovulation treatment can be expected. At a standard dose some animals ovulate more oocytes than the fimbriae can efficiently pick up, others do not superovulate at all. In general, the higher the gonadotropin dose level, the greater the response, but also the greater the variability in response among animals. Besides individual variability, other factors that affect superovulation rates are the route of gonadotropin administration, breed of animal, state of health and nutritional plane, age of the animal, and whether or not she has been superovulated previously. High doses of gonadotropin may also result in a hormonal environment adverse to fertilization. Some superovulated cows fail to show estrus, but when normal estrus occurs, insemination can be either natural or artificial. Some reports indicate better fertility with fresh than with frozen semen.

## 2 Embryo Collection and Transfer

As many variations in techniques exist for embryo collection and transfer as for superovulation procedures. While research continues on developing nonsurgical collection and transfer techniques for cattle, including collection and transfer through the cervix, the most successful results to date have been obtained using surgical procedures. Usually embryos are collected 4–6 days after estrus. The embryo is in the eight-cell to early morula stage at this time and has recently reached the uterus. A midventral incision with the donor under general anesthesia is the most common procedure for embryo collection. Some procedures involve cannulation of the oviduct and flushing a tissue culture medium retrograde through the uterus and oviduct and out the cannula. Others involve flushing the embryo directly from the

uterus. The embryo is observed microscopically for morphological normality, drawn into a sterile pipette, and transferred to the uterus of the recipient female. Either a midventral incision under general anesthetic or flank incision under local anesthetic is used for transfer of the embryo to the recipient. Complete asepsis is important throughout the collection and transfer procedure.

### 3. Synchronization Requirements

It is important that the developmental stage of the embryo is closely synchronized with the luteal phase and endometrial differentiation of the recipient female. Work with sheep indicates that  $\pm 2$  days synchronization of donor and recipient estrus may be adequate. Work on a limited number of cows (86) suggests synchronization requirements are more exacting with only  $\pm 1$  day variation giving acceptable fertility. If a large pool of recipients is available, animals whose estrus is synchronized with the donor can be selected. Prostaglandin or progestagen administration can also be used to synchronize estrus of the recipients with the donor without an apparent decrease in fertility (91). Another technique that has potential use, but is not currently used, is cooling the embryo below 20°C to halt mitosis and development of the embryo. When available recipients reach the luteal stage corresponding to the developmental stage of the embryo, the embryo is rewarmed and transferred.

### B. SUCCESS OF EMBRYO TRANSFER

Successful embryo transfer experiments have been reported by L. E. A. Rowson and co-workers (84-86). Initial reports based on a limited number of females indicated as many as 91% of the recipients became pregnant following embryo transfer. Since these results were published, a number of workers have reported pregnancy rates ranging from 0 to 92% (16, 36, 89, 90, 91). Many of these results, including those of Rowson *et al.*, are confounded by the transfer of more than one embryo to each recipient. Many groups report that, on the average, 50-75% of the recipients become pregnant following transfer. It should be noted, however, that the percentage of recipients that become pregnant may not be the best means for determining transfer efficiency. For example, the number of calves born from each superovulated donor may provide a better measure of potential benefits to a livestock producer. Though additional research is needed to improve many phases of this exciting procedure, it may be an important tool for genetic improvement of domestic animals.

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# 12 Implantation and Development of the Conceptus

P. Eckstein and W. A. Kelly

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## I. Introduction

In the following description of implantation and fetal development in domestic mammals, attention has been focused throughout on recent advances in this areas

As a rule, no attempt is made to deal with intricate morphological or ultrastructural detail except in the case of the placenta, where greater knowledge contributes significantly to an understanding of function. For information on comparative anatomical, specific embryological, and more general physiological aspects of these subjects the reader is referred to the many excellent and comprehensive accounts available in the literature, such as Amoroso (5), Nalbandov (68), McDonald (66), Hamilton, Boyd, and Mossman (50), Sack (77), and Wimsatt (90), as well as to the proceedings of a recent symposium on "Equine Reproduction" (38).

The chapter is confined to the situation in the common domestic animals, that is, the sheep and cow (among ruminants), the mare and pig (non-ruminant ungulates), and the bitch and cat (carnivores), but does not deal, or only occasionally so, with other representatives of these orders.

## II. Anatomy of the Uterus

The gross anatomical features of the reproductive organs in domestic animals have been repeatedly described [e.g., Eckstein and Zuckerman (37), Nalbandov (68), McDonald (66), Sack (77), and Hafez (48)]. A diagrammatic comparison of the female reproductive tract is shown in Fig. 1.

The uterus is bicornuate and communicates with the exterior through a single cervix and vagina. The two uterine horns show, however, variable degrees of external and internal fusion, and corresponding variation in the size and configuration of the common uterine cavity. In ruminants, in

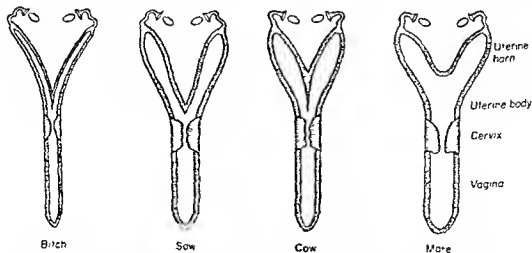


FIG. 1. Comparison of the female reproductive tract in the domestic animals (diagrammatic). Modified from Nickel *et al.* (77).

spite of considerable external fusion, this common cavity is minute, the two uteri remaining separated by a thin median septum which nearly reaches the cranial end, or internal os, of the cervical canal (see Fig. 1). In the sow this septum is shorter, with a consequent increase in the size of the common cavity, in spite of marked external separation of the horns. In the mare there is no trace of a midline septum, and the size of the internal cavity corresponds to the externally fused part of the uterus and forms a distinct single "body," except at its upper, paired extremities.

The cervix is a conspicuous, fibrous segment in all the domestic animals, except carnivores. In ruminants it is provided with a series of hard, conical ridges or annular rings. These are less prominent in the sow and absent in the mare, bitch, and cat. In the latter, the cervix is directly continuous with the posterior wall of the vagina, without forming a proper "portio" and vaginal fornices.

### III. Tubal Passage, Spacing, and Attachment of Ova

Following fertilization (see Chapter 11) and tubal passage (see below, Section III,A), the embryos enter the uterus and, after a variable period of "free life" in its lumen, begin to attach to the endometrium. In carnivores this is followed by implantation and formation of a feto-placental connection. In other domestic species, however, the intimacy of contact between the fetal and maternal tissues varies.

During the preattachment phase the developing conceptus is nourished, first by its own yolk substance and the tubal fluids, and then by the secretion of the uterine glands, the so-called uterine milk (see Section VII). Once formed, the placenta becomes chiefly responsible for maintenance and growth of the embryo.

In all the domestic animals the conceptus remains within the uterine cavity and expands to fill its lumen; this is referred to as "central implantation." In other types of mammals implantation may be eccentric or interstitial (cf. 21).

#### A. TUBAL PASSAGE

Tubal passage of the ova in domestic animals is relatively constant, as in other mammals, and generally lasts about 3 to 4 days (17, 21). The pig, however, is an exception since in this species fertilized eggs enter the uterus approximately 48 hours after their release from the ovary. This has been established both in naturally ovulating sows and in others in which the time of ovulation had been accurately controlled by HCG (55). The pig embryo

reaches the uterus at or shortly after the four-cell stage (55). Its exceptionally rapid passage through the tube may be related to the regular occurrence of multiple ovulations in the pig. It does not, however, appear to be due to excessive secretion of estrogen or progesterone since their concentrations were not significantly raised by HCG treatment and that of progesterone is almost undetectable at ovulation during the normal cycle (43, 56).

In the mare, normal tubal transport appears to depend on the occurrence of fertilization. Unfertilized eggs are retained in the oviduct (13).

## B. SPACING AND ATTACHMENT OF EGGS

Upon arrival in the uterus, the eggs of domestic animals are distributed through the two horns. In the ewe and cow, in which usually only one egg is shed, it becomes attached in the distal part of the horn adjacent to the ovulating ovary; in the mare it frequently moves to the opposite horn. When two eggs are released from a single ovary in the sheep, one generally migrates and attaches in the contralateral horn (21). In the polytocous sow, the eggs are evenly spaced between the two uterine horns (35). The mechanisms concerned in the spacing of blastocysts have been reviewed by Böving (20).

## C. OVUM IMPLANTATION

Attachment of the blastocyst (or "chorionic vesicle") in the farm animals is, unlike that in most higher mammals, a relatively slow and gradual process. In the sow it consists of the microvilli on the surface of the chorion interdigitating with corresponding ones on the maternal epithelium. The conceptus is, therefore, held in the uterus merely by the adhesiveness of the microvilli. Attachment begins between days 14 and 16 of gestation, with the alignment of the chorion and uterine epithelium, and the first definitive interdigitations can be observed on day 18 (29).

In the ewe, too, the embryo begins to attach during the 15th day of gestation, when the chorionic vesicle has grown sufficiently to come into close contact with the entire uterine epithelium of the horn (19). True interdigitation of the embryonic and maternal tissues follows early in the fourth week.

In the mare complete attachment of the chorion to the endometrium may not occur over the whole conceptus until as late as days 90-95 of gestation (5).

Throughout the preimplantation stage, between arrival of the embryo

in the uterus and its attachment, the conceptus should not be regarded as a "passive object." Thus in sheep, regression of the corpora lutea of the estrous cycle is inhibited even before close contact between the chorion and the endometrium is established. The agent responsible for the maternal "recognition" of pregnancy and maintenance of the corpora lutea is believed to be produced by the conceptus itself (52; see Section III,E).

The time of implantation in the cow is given as between the second and fifth week of gestation (24, 90). In the bitch and cat it is thought to occur between the end of the second and the third week after mating (5, 54).

By contrast, in some close relatives of the domesticated animals nidation is constantly and markedly delayed. For instance, in roe deer, among ruminants, the "free vesicle" stage lasts for about 5 months, and in the badger, a carnivore, nearly a year (90).

#### D. UTERINE ADAPTATIONS FOR IMPLANTATION

All the species considered here, except the cat, ovulate spontaneously and therefore develop functional corpora lutea during their unmated cycles. Hence, the changes in the uterus in the early stages of pregnancy are those of the luteal phase of the estrous cycle, namely, an increase in the vascularity of the endometrium and an increase in glandular growth and activity.

In the pig, cow, and sheep the outer, trophoblastic layer of the blastocyst is noninvasive, and there is no pronounced decidual reaction in these species comparable to that found in rodents. In the carnivora the trophoblast penetrates the maternal tissue, and the endometrium becomes decidualized.

The early gestational stages in the horse are characterized by a special feature, the appearance of the so-called endometrial cups. These number about a dozen and are present from about the sixth to the twentieth week of pregnancy. They are distributed in roughly circular fashion around the attachment of the yolk sac. Endometrial cups are also found in the donkey and zebra and in the mare carrying a mule fetus.

In the past it was thought that the endometrial cups arose as modifications of the endometrium in the fertile uterine horn in response to the trophoblast (cf. 2nd edition, Chapter 14, p. 389). It is, however, now established that the cups, in fact, are constituted by specialized trophoblast cells which invade the maternal endometrium (3, 4, 49; Fig. 5). This finding is supported by the fact that fetal genotype affects the concentration of PMSG in maternal blood (4, 25, 38). In addition, it has now been demonstrated that cells of the allantochorionic girdle, derived from the fetal chorion, can synthesize large quantities of PMSG in tissue culture

(4, 38). Some quantitative aspects of PMSG secretion in the mare are described in Chapter 15.

The endometrial cups begin to slough off after about the twentieth week of pregnancy which coincides with a marked reduction in the secretion of PMSG. The destruction of the endometrial cup tissue is accomplished by an invasion of lymphocytes and, according to some workers, resembles an immunological rejection process (52).

In ruminants growth of placental tissue only occurs in relation to specialized areas of the endometrium (cotyledons or caruncles; see Section V). Uterine changes during pregnancy consist mainly of a pronounced increase in the vascularity of the cotyledons; in the intercotyledonary areas there is a marked development of the uterine glands.

In the pig regional differences in the differentiation of the endometrium appear by the 22nd day of gestation. Near the developing allantois the endometrium becomes hyperemic, while the areas adjacent to the poles of the chorionic vesicle become very pale and translucent (40).

In the bitch and cat, too, there is a progressive development of the uterine glands and crypts and the endometrium becomes hyperemic. In the former, local edema can be observed at the definitive implantation site of each embryo during days 17-18 post coitum (p.c.) (54).

## E. OVUM TRANSFER AND LUTEOLYSIN

Research during the past decade has transformed previous views about the factors involved in the experimental transplantation of embryos and the role of the luteolytic principle in controlling activity of the corpus luteum.

Successful ovum transfer in mammals depends on perfect correspondence between the age of the transplanted embryo and that of the recipient endometrium. This was first shown by Chang (23) in the rabbit and by Noyes and Dickmann (69) in the rat, and has since been demonstrated also in the sheep by Rowson and Moor (75). Thus in sheep, exact synchrony of donors and recipients resulted in a conception rate of 75% in the host ewes; when the donor and recipient differed by  $\pm 3$  days, only 8% conceived (75).

The existence and physiological role of luteolysin has been convincingly demonstrated in several domestic animals such as the pig, sheep, and cow, as well as in the guinea pig (11, 74, 79), and the vascular pathways that may be involved in conveying it have been described in the sow and cow (33). The agent concerned is thought to be elaborated within the uterus and to be responsible for terminating the functional life of the corpus luteum of the cycle (11). Although it has not yet been unequivocally

isolated from the endometrium, assays of uterine venous effluent in sheep (86) and guinea pigs (18) strongly suggest that the principle is of uterine origin and identical with prostaglandin  $F_{2\alpha}$  (see also Chapter 4).

The preservation and conversion of the corpus luteum of the cycle into that of pregnancy in sheep has been ascribed by Moor and Rowson (67, 74) to an active influence exerted by the early embryo. For instance, removal of the sheep embryo before the twelfth day of gestation leads to regression of the corpus luteum at the time usual for the cycle, the ewe remaining effectively unaware of having conceived. Alternatively, transfer of a 12- to 13-day-old embryo into a nonpregnant sheep causes the corpus luteum to be maintained and to be converted from the cyclic type to that of gestation. The available evidence suggests that the embryo of the sheep, even before its attachment to the endometrium, exerts an active antiluteotropic effect and so prevents the regression of the corpus luteum which occurs normally during the cycle (74).

#### IV. Nature and Origin of the Extraembryonic Membranes

The extraembryonic, or fetal, membranes are developed from the zygote, but form no part of the embryo itself. They protect and serve in nourishing the embryo, and are shed at birth. There are four membranes, the amnion, chorion, yolk sac, and allantois, generally found in reptiles, birds, and mammals; placentation has, therefore, not led to the evolution of any extraembryonic structures not already present in egg-laying vertebrates such as the hen. The process of formation of the extraembryonic membranes in the domestic animals is illustrated in Figs. 2-5. [In these drawings, the extraembryonic membranes are generally shown as single lines and, therefore, do not illustrate the basically bilaminar (i.e., ectodermal or endodermal plus mesodermal) nature of these structures.]

##### A. AMNION

The amnion of ungulates and carnivores is formed by folding, as in the chick. Circumferential folds of extraembryonic ectoderm and somatic mesoderm grow up around the embryo and then meet dorsally to enclose it completely in a fluid-filled amniotic sac which is lined by ectoderm (Figs. 2-5).

##### B. CHORION

This constitutes the outermost of the extraembryonic folds. It is derived from the outer wall of the blastocyst, or trophoblast, and the term "chorion"



is applied to it after the amnion has formed (see Figs. 2-5). The ectoderm of the chorion is continuous with that of the embryo, and becomes lined with somatic ("somatopleuric") mesoderm.

Both the amnion and chorion originate from ectoderm and somatopleuric mesoderm and are, therefore, nonvascular. The yolk sac and allantois, by contrast, develop from endoderm and splanchnopleuric (vascular) mesoderm, and are, thus, potentially capable of forming functional placentas after establishing contact with the uterus.

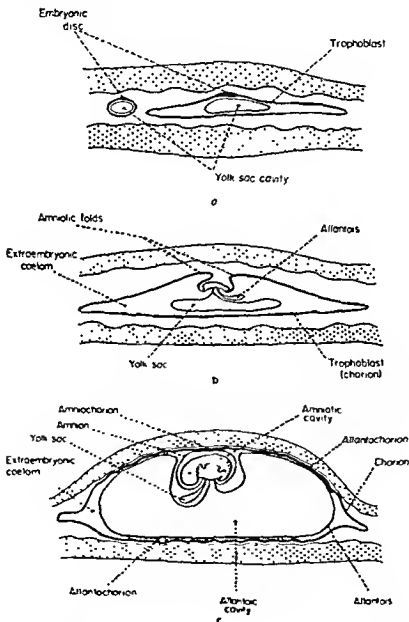


FIG. 2. Formation of the fetal membranes in the pig. (a, b) Free-vesicle stage; (c) after attachment to endometrium. Based on Mossman (67a) and Patten (72a).

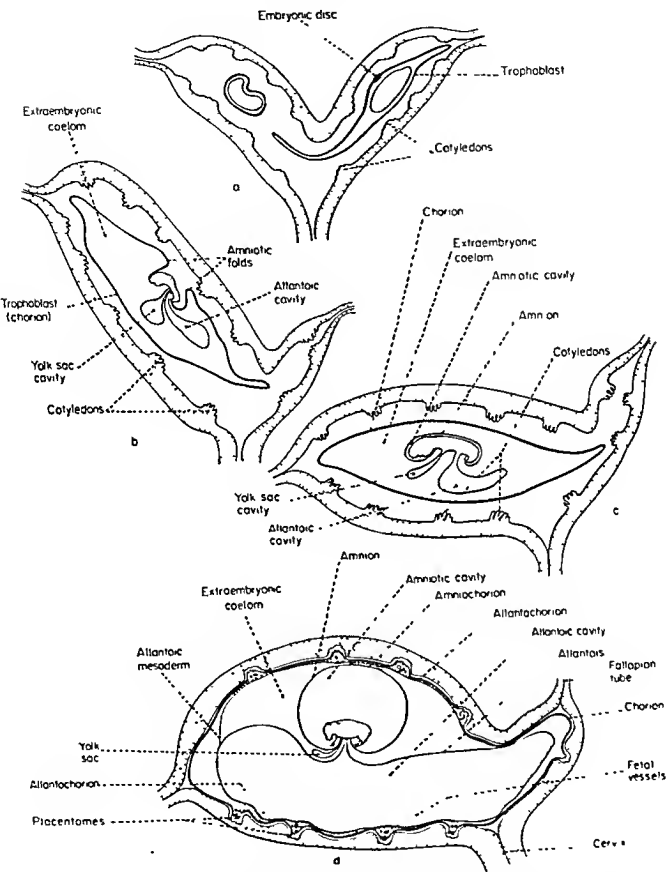


FIG 3. Formation of the fetal membranes in the sheep (a-c) Free-vesicle stage; (d) after attachment to endometrium. Based on Mossman (67a) and Boyd and Hamilton (21).

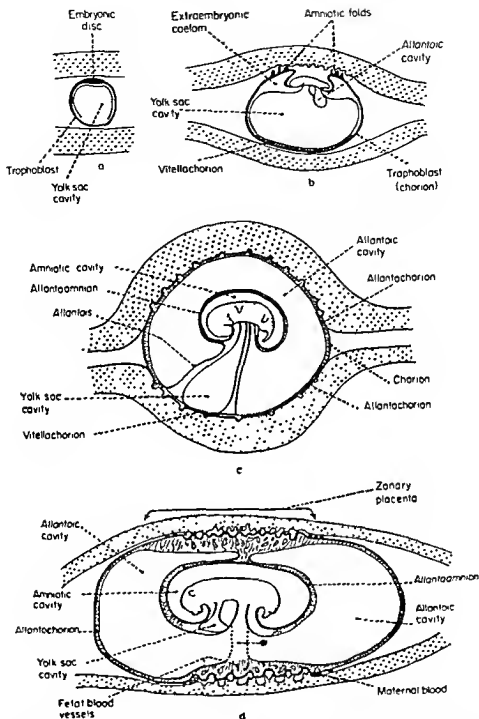


FIG. 4. Formation of the fetal membranes in the bitch. (a, b) Free-vesicle stage; (c) after attachment, (d) after formation of placenta; \*: course of allantoic vessels. Based on Hamilton *et al.* (49a).

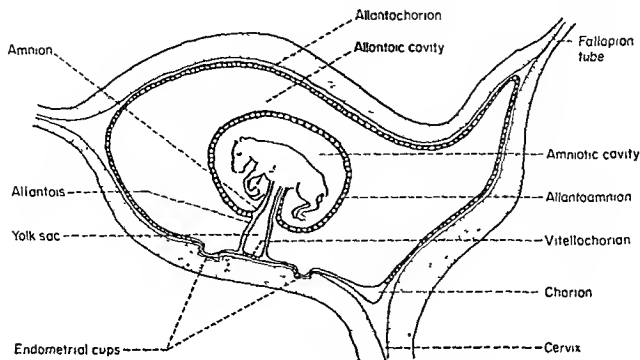


FIG. 5. Appearance of the fetal membranes in the mare at about the third month of gestation Modified from Amoroso (5) and Allen and Moor (4).

### C. YOLK SAC

The yolk sac is formed from extraembryonic endoderm spreading over the inner surface of the trophoblast in the midgut region. The bilaminar structure becomes the trilaminar yolk sac by the insinuation of splanchnic mesoderm between the trophoblast and endoderm; when vascularized, it constitutes the vitellochorion (Fig. 4b,c).

### D. ALLANTOIS

This forms as an outgrowth of the hindgut. It is covered by splanchnic mesoderm in which the allantoic vessels develop. The allantois is particularly well developed in ungulates, but there is great variation in the relative size, degree of fusion, function, and persistence of all the extraembryonic membranes.

To establish an organ of interchange between mother and fetus, the outer chorion must be vascularized by an extraembryonic circulatory system. This may be provided by either the yolk sac vessels or the allantoic ones. If it is vascularized by the vitelline vessels, a vitellochorion and, after contact with the endometrium, a yolk sac-type of placenta results. Alternatively, if vascularization is by the allantoic vessels, an allantochochion and, consequently, an allantochochial placenta is formed.

In eutherian mammals the allantochochial placenta is the chief form of placentation and the yolk sac placenta is usually only transitory. A true yolk sac-type of placenta only develops in the mare and carnivores (see

Figs. 4c and 5). In the pig and ruminants the yolk sac does not make close contact with the maternal tissues (see Figs. 2 and 3), but is believed to play a part in the nutrition of the early embryo by absorbing "uterine milk" (see Section VII,A).

The extraembryonic membranes develop with great rapidity during the first half of pregnancy. In the pig they grow faster than the fetus up to about day 65, but from then on their development falls behind that of the fetus (73). The allantoic fluid increases rapidly to a maximum from the third to the ninth week and then decreases as swiftly. According to McCance and Dickerson (65), the initial source of the fluid is the secretory activity of the allantois itself, and the fetal kidney only begins to contribute to it in later stages of pregnancy (1).

In the sheep it is believed that expansion of the allantois and the accumulation of fluid within it can be related to the activity of the fetal mesonephros as early as day 18 of gestation (31).

Fetal urine passes into the allantoic sac via the urachus until about the end of the third month. Subsequently, it drains increasingly into the amniotic sac, presumably because of occlusion of the urachus and greater patency of the urethra (2).

## V. Growth of the Conceptus and Formation of the Placenta

Among the domestic animals, early development of the conceptus is best known in the sheep and pig.

In the sheep, the embryo enters the uterus 66 hours after ovulation at the eight-cell stage of development (53). The zona pellucida is generally shed by the seventh to eighth day (44, 76), and attachment of the blastocyst is believed to be brought about by loose "surface-to-surface contact" (19). During this phase the conceptus elongates and grows with astonishing speed. Rowson and Moor (76) state that from the twelfth day post-estrus (or some 11 days after ovulation and fertilization) it changes within 24 hours from a blastocyst about 1 mm in diameter to an elongated chorionic sac with a mean length of 11.7 mm; it reaches a length of over 100 mm on day 14 (Fig. 6). The embryonic disc itself, however, grows only slightly during the same time. Between days 15 and 17 p.c. the speed of growth of the conceptus increases even more (76; Fig. 6).

Apart from its phenomenally rapid rate of growth, the sheep conceptus begins to exert, as previously mentioned, a hormonal influence upon the corpus luteum and, after the twelfth day postcoitum, to be responsible for its conversion into the corpus luteum of pregnancy (67; see also Section III,E).



FIG. 6. The blastodermic vesicle of the sheep on day 14 after estrus (total length 112 mm). (Reproduced from Rowson and Moor, 76.)

The placenta in ruminants is cotyledonary or multiplex, that is, it is formed by the fusion of rounded projections of the uterine mucosa, the so-called caruncles or maternal cotyledons, with corresponding localized tufts of chorionic villi, the fetal caruncles, to form functional units known as "placentomes" (5). In the sheep the fetal caruncles appear on about the 22nd day, when fusion of the mesoderm surrounding the rapidly expanding allantois with that of the chorionic sac is complete (32). The wall of the chorionic vesicle begins to adhere to and eventually fuses with the maternal cotyledons, in this way forming placentomes. The maternal aspect of the placentome is concave in the ewe and convex in the cow (Fig. 7). The intercotyledonary or membranous portion of the chorion takes no part in the formation of placentomes.

Later, elongated slender chorionic villi, some 10 mm in length, develop and become attached to pits, or "crypts," within the endometrial stroma. The placentomes of the sheep increase in size and number until there are 90-100 on about the 90th day, after which they begin to diminish in size and probably number. According to Hammond (51), development of the placentomes in the cow is not complete until the third or fourth month of pregnancy.

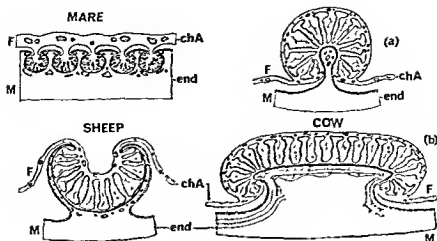


FIG. 7. Structure of the definitive placenta of the mare, Welsh Mountain sheep, and Jersey cow. F, fetal; M, maternal; chA, chorioallantois; end, endometrium. (a) Transverse section; (b) longitudinal section. (Reproduced from Silver *et al.*, 83.)

In the pig, early development and attachment of the ovum resemble those in the ewe and cow (28, 45, 64). The relations between the fetal and maternal tissues remain very simple. No caruncles, and, hence, no placentomes, are present as in ruminants, and the pregnant uterine mucosa retains much the same structure as during the estrous cycle. The mouths of the endometrial glands are covered by minute circular collections, or domes, of trophoblastic cells, the so-called areolae, which are thought to be involved in absorption of uterine milk. The chorion, with the allantois closely applied to its inner surface (see Fig. 2c), is in loose contact with the uterine epithelium. It can be easily detached throughout gestation by gentle traction, and at parturition the fetal membranes separate from the uterine tissues without lesion or hemorrhage, leaving behind an intact surface. Initially the uterine mucosa is smooth but after about the second week the epithelium becomes pitted. At the same time the chorion develops corresponding vascular folds or ridges [though not true villi (5)], which fit into the depressions of the mucosa and promote attachment and functional contact. The primitive yolk sac forms the first placental connection with the maternal tissues in the pig. It is quickly superseded by the vascular allantois, which expands rapidly after the third week and fuses with the chorion. Eventually, the extremities of the allantochorion become constricted into atrophic tips.

As in the sheep, the elongated and wrinkled chorionic vesicle of the pig grows with extreme rapidity during this early phase of development. According to Corner (27), by the 17–18th day the uterine cavity is completely filled with embryonic vesicles measuring 30–40 cm.

There appears to be far less information on growth of the conceptus in domestic carnivores. Some observations on fetal growth in these animals and in the horse and cow are referred to below (Section VII,C).

The time around implantation represents one of the most critical stages of gestation. For instance, in the pig the bulk of prenatal mortality, estimated variously at between 30 and 40% (28, 73), is believed to have occurred by the end of the third week of pregnancy (73).

## VI. The Placenta and Ultrastructure of the Feto-Maternal Junction

The function of the placenta is to transfer nutritive material and oxygen from the mother to the fetus and excretory products in the opposite direction.

All types of placentas are composed of a maternal element, the endometrium of the uterus, and on the fetal side, the chorion. Hence, most simply defined, placentation means the apposition or fusion of the chorion with the endometrium for physiological exchange.

The definitive placenta of the domestic animals, like that of all higher mammals, is of the allantochorial type, that is, it is formed by the fusion of the vascular allantois with the chorion. The placenta may be classified in a number of ways, such as by its gross shape, or the distribution ("zonary," "diffuse," etc.) of chorionic villi. Grosser (46, 47) classified the placenta according to the number of tissue layers intervening between the fetal and maternal blood, and recognized four principal types.

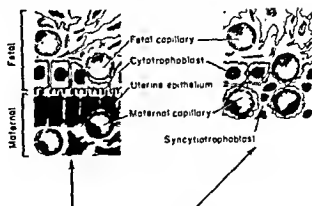
In the first, the epitheliochorial placenta, present in the mare and sow, there is no erosion of maternal tissues and the allantochorion is opposed to the endometrial epithelium (Table I). In the second, the syndesmochorial placenta of the sheep, cow, and goat, the maternal epithelium is lacking and the fetal chorion is in contact with the endometrial stroma. In the endotheliochorial placenta of the cat and dog there is contact between the chorion and the endothelium of the maternal blood vessels (Table I), while in the fourth type, the hemochorial placenta of man, nonhuman primates, and rodents, the chorionic villi are suspended in a pool of maternal blood.

Grosser's classification, based on a purely histological analysis of the tissues separating the fetal chorion from the maternal blood, was of great practical value and correspondingly popular, but must now be looked on as an oversimplification (see below, Section VII,A). Several of Grosser's morphological findings have also been superseded by the results of more recent ultrastructural investigations. These indicate that the maternal epi-



TABLE I

The Feto-Placental Junctional Zone in Different Types of Placenta\*



Type of placenta	Epitheliochorial	Endotheliochorial	Hemochorial
Maternal tissue			
Endothelium	+	+	-
Epithelium	+	-	-
Fetal tissue:			
Trophoblast			
Cytotrophoblast	+	+	+
Syncytiotrophoblast	-	+	+
Endothelium	+	+	+
Examples:	Horse, pig, cattle	Cat, dog	Man, monkey

\* Insets show the relationships between the fetal and maternal tissues, as revealed by electron microscopy, in the epitheliochorial and endotheliochorial placenta (modified from Hamilton *et al.*, 50).

thelium persists in the definitive ruminant placenta (16), and the latter must, therefore, be classified as epitheliochorial rather than syndesmochorial. Since the placenta is an organ for metabolic exchange, its efficiency will depend on its surface area. In all species with an epitheliochorial type of placenta so far studied by electron microscopy, interdigitating microvilli have been found on the surface of the chorion and the uterine epithelium. Such interdigitation of microvilli greatly increases the surface available for interchange. Furthermore, ultrastructural studies have confirmed the existence of intraepithelial capillaries, thus ensuring that the maternal and fetal circulations are brought into the closest possible contact (Table I).

The microcirculation within the placenta is now considered to be a very important factor in gaseous exchange. In this connection, it may be

significant that in both the mare and cow the fetal and maternal capillaries are in close, intraepithelial contact. By contrast, in the ewe there is a relatively wide boundary zone, formed by the syncytial border of the maternal epithelium, which separates the maternal and fetal circulations (83; see also Section VII,A).

Again, the distinction between the diffuse and the cotyledonary type of placenta is not as sharp as was once thought. In the mare, the surface of the placenta is increased by small tufts of chorionic villi which project into corresponding invaginations of the endometrium forming microcotyledons. According to Samuel *et al.* (78), microcotyledons can be recognized by the 60th day of gestation as a simple folding of the trophoblast and uterine epithelium. The folding process continues and by the 150th day of gestation, the microcotyledons are well developed.

In the cow and sheep there is a microvillous junctional zone between the epithelium lining the crypts and that covering the chorionic villi. The cryptal lining is cellular in the cow, but syncytial in the sheep.

At parturition in the cow the placenta separates within the cotyledons at the line of microvillous attachment, with little damage to either the fetal or maternal tissues. By contrast, in the ewe, the placenta separates as a result of very rapid degeneration of the chorionic epithelium in the short interval between parturition and delivery of the fetal membrane (85). In the ewe retention of the placenta is very rare, while in the cow this is a common clinical condition, a difference which may be explained by the dissimilar methods of separation of the fetal and maternal tissues in both species.

In carnivores the trophoblast differentiates into an outer syncytiotrophoblast in contact with the maternal tissue and an inner cytotrophoblast. A common feature of the feto-maternal junction in these animals is the reduction of the maternal epithelium and connective tissues, although there are minor species differences. The syncytiotrophoblast is intimately fused with a substance or membrane of disputed origin and occasionally with the maternal endothelium. The membrane has been considered by some workers to be a basement membrane of maternal endothelial origin (16). As the trophoblast is so firmly attached to the maternal endothelium by the intervening membrane no separation of the tissues is possible at parturition. Instead, maternal tissue, the decidua, is lost with the fetal placenta.

In both the bitch and cat the endotheliochorial nature of the placenta has been substantiated by electron microscopy (9, 10, 34). The enlarged endothelial cells of the maternal capillaries have a spongy cytoplasm and

are separated from the trophoblast by an incomplete basement membrane, so that in some regions the plasma membrane of the endothelial cells may be in contact with the syncytial trophoblast.

## VII. Fetal Nutrition and Growth

### A. FETAL NUTRITION AND PLACENTAL FUNCTION

The two main sources of nutrition for the developing embryo are "histotroph" and "hemotroph," which together constitute the "embryotroph."

Histotroph is a milky fluid (more commonly referred to as "uterine milk") containing the secretions and debris of the endometrial glands plus extravasated maternal blood. Uterine milk is secreted into the uterine lumen and absorbed by the fetal membranes.

Hemotroph consists of nutritive materials absorbed directly from the circulating maternal blood by the allantochorion or vitellochorion, where it is in contact with the uterine tissues.

The eggs of placental mammals contain very little yolk. The developing conceptus, therefore, depends on external sources of nutrients from the beginning of intrauterine life. In all types of mammal, histotroph is essential before the establishment of the placenta, and in most domestic animals, especially the pig, horse, sheep, and goat, remains so throughout the greater part of pregnancy.

Most experimental work on the nutritive function of the definitive allantochorial placenta has been centered on the hemotroph, largely ignoring the histotroph, and has been dominated by Grosser's classification (see Section VI) and its implications on placental permeability. It was generally assumed that the transfer of nutrients occurs by a process of diffusion and that there is a gradient from a high concentration in the maternal vessels to a lower one in the fetal vessels. Grosser's system of classifying placentas according to the layers separating the two circulations fitted in naturally enough with this concept, the epitheliochorial type being considered more primitive and inefficient as an organ of exchange than the endotheliochorial and hemochorial types. The fallacy of this interpretation was exposed by Barcroft (12), when he emphasized the high degree of maturity of the newborn foal and calf compared with the extreme immaturity of the human baby or rodent at birth, and concluded that there could be nothing physiologically inadequate about an appositional, epitheliochorial placenta.

Subsequent work has shown that the physiological "efficiency" of the placenta in the domestic animals may be determined by factors such as

the placental perfusion rate and metabolism in addition to the permeability of the placental barrier. Thus the higher transfer rates of glucose, free fatty acids, and lactic acid across the placenta of the mare as compared with those in ruminants may be related to the shorter intercapillary distance between mother and fetus in the equine placenta (83).

The distinction between histotroph and hemotroph is not always clear-cut. When studying the ultrastructure of the placental labyrinth in the ferret, Lawn and Chiquoine (61) established that secretions transmitted from the mother to the fetus are synthesized in the endothelium of the maternal blood vessels in much the same way as nutritive material is secreted by the uterine glands. Hence, what had been assumed to be hemotroph was, by definition, histotroph.

## B. RESPIRATION

Apart from the transfer of nondiffusible nutrients from mother to fetus and of excretory material in the opposite direction, the placenta also acts as an organ for gaseous exchange.

Diffusion of oxygen and carbon dioxide is generally believed to occur in the areas of the allantochorion in which the maternal and fetal circulations are in closest proximity, e.g., in ruminants gaseous exchange takes place in the cotyledons and not in the intercotyledonary areas.

Silver *et al.* (83) and Comline and Silver (26) have shown that there is a correlation between the specialized vascular architecture of the placenta and its efficiency in gas exchange. In the microcotyledons of the mare, the maternal and fetal capillaries are arranged in parallel, in a countercurrent fashion. In the placentome of the sheep, the maternal vessels are in the form of a cascade of capillaries which permits a considerable venous admixture within the system, and must, therefore, be considered a less efficient system.

The sheep placenta attains its maximum size after 60 to 70 days (12), while growth of the fetus is most rapid in the last stages of gestation.

Comline and Silver (26) consider that the way in which the blood flow through the uterus is closely adjusted during pregnancy to fetal rather than placental weight is one of the most important unsolved problems of placental exchange.

## C. FETAL GROWTH

In spite of their increasing use in experimental work, the prenatal development of domestic animals has received comparatively little attention.

are separated from the trophoblast by an incomplete basement membrane, so that in some regions the plasma membrane of the endothelial cells may be in contact with the syncytial trophoblast.

## VII. Fetal Nutrition and Growth

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Hemotroph consists of nutritive materials absorbed directly from the circulating maternal blood by the allantochorion or vitellochorion, where it is in contact with the uterine tissues.

The eggs of placental mammals contain very little yolk. The developing conceptus, therefore, depends on external sources of nutrients from the beginning of intrauterine life. In all types of mammal, histotroph is essential before the establishment of the placenta, and in most domestic animals, especially the pig, horse, sheep, and goat, remains so throughout the greater part of pregnancy.

Most experimental work on the nutritive function of the definitive allantochorial placenta has been centered on the hemotroph, largely ignoring the histotroph, and has been dominated by Grosser's classification (see Section VI) and its implications on placental permeability. It was generally assumed that the transfer of nutrients occurs by a process of diffusion and that there is a gradient from a high concentration in the maternal vessels to a lower one in the fetal vessels. Grosser's system of classifying placentas according to the layers separating the two circulations fitted in naturally enough with this concept, the epitheliochorial type being considered more primitive and inefficient as an organ of exchange than the endotheliochorial and hemochorial types. The fallacy of this interpretation was exposed by Barcroft (12), when he emphasized the high degree of maturity of the newborn foal and calf compared with the extreme immaturity of the human baby or rodent at birth, and concluded that there could be nothing physiologically inadequate about an appositional, epitheliochorial placenta.

Subsequent work has shown that the physiological "efficiency" of the placenta in the domestic animals may be determined by factors such as

Systematic studies of fetal growth in homogeneous populations or single breeds, and using both sufficient numbers and adequate techniques, appear to have been carried out mainly in the sheep (59, 87) and pig (73), but only rarely in the other domestic animals. Recently, however, Evans and Sack (39) have compiled summaries of normal embryonic and fetal development in domestic and laboratory mammals, based on the literature and on specimens in the collection of embryos at Cornell University, New York. Their report includes growth curves and chronological tables of external features of the developing dog, cat, cow, and horse, and is a significant source of information on embryonic and fetal growth in these species. Morphological changes in the equine fetus and placenta at known stages of gestation have also been described by Douglas and Ginther (36).

In the fetal sheep, length (forehead-rump, F-R) increases much more regularly than weight, and in the opinion of authors such as Barcroft (12), over the last two-thirds of pregnancy, F-R length grows in an almost linear relation to fetal age, with little difference between single and twin lambs. The view that fetal growth in length proceeds in linear fashion is not shared by other workers, who believe that it more nearly approximates an elongated S, or double-parabolic type of curve.

There is, however, complete consensus that weight is far more variable and provides a less reliable index of fetal growth than length. Variability is most pronounced throughout the last one-third of gestation, and during this phase the time-weight curve constructed from the means of the whole litter bears little relation to the growth of individual young. Why some fetuses appear to falter at this stage of prenatal growth while others do not is unknown and requires further study. Competition for food with others in the litter appears to be one of the most important factors, and it is well established that an increase in litter size is associated with a decrease in fetal and birth weight in pigs and sheep (72).

Prenatal development of cattle is very similar to that in sheep (39, 44, 72; Fig. 8A). In these animals absolute weight increases little during the first half of intrauterine life, and the greatest gain in weight is made in species during the last third of pregnancy.

In the horse, fetal growth, whether assessed as crown-rump (C-R) length (Fig. 8B) or as the cube root of weight (cf. Cole and Cupps, 2nd edition, Fig. 11, p. 408), shows an essentially linear relation to age.

The pronounced effect of prenatal influences on the size of the newborn foal was strikingly demonstrated by the reciprocal Shetland-Shire crosses carried out by Walton and Hammond (88). At birth the crossbred foal of the Shire dam was about three times as large as that of the Shetland mare.

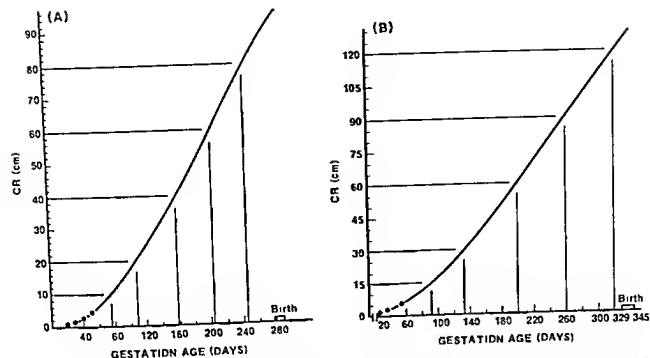


FIG. 8. (A) Fetal growth in the cow. (Reproduced from Evans and Sack, 39.)  
(B) Fetal growth in the horse. (Reproduced from Evans and Sack, 39.)

### VIII. Chorloallantoic Anastomoses and Twinning

In multiple gestation in the cow there is usually fusion of the chorio-allantoic blood vessels of adjacent fetuses; the fused vessels may have a diameter of 1 cm. When, in twin pregnancy, both fetuses occupy the same horn, fusion and vascular anastomoses occur in every case, and when one is in each horn, their incidence is 67% (89). The presence of common vascular channels between the fetuses is associated with the development of immunological tolerance of one twin for the other; an example of this is the freemartin.

The origin of the term "freemartin," is obscure. According to Forbes (41), "free" may be a contraction from the Anglo-Saxon "faer" (empty, void) or Scottish "ferow" (barren, not carrying a calf), while the Gaelic "mart" or "martin" probably signified a spayed heifer or cow selected for slaughter because of infertility (cf. also Short, 80).

If cattle twins are of unlike sex and have a common placental circulation, the male is phenotypically normal and the female shows partial sex reversal into a sterile freemartin. This is characterized by atrophy of the presumptive ovary and the retention of some medullary tissue in the gonad, resulting in the regression of the Müllerian duct and development of the Wolffian duct and its derivatives (80, 81). Over 90% of all females of heterosexual cattle twins are freemartins (14).

Following the classic description of Keller and Tandler (60) and Lillie (62, 63), it was widely believed that the freemartin state is brought about by "androgen," which is conveyed from the male twin through the vascular connections to the female fetus and affects its reproductive tract during the indifferent stage of development. Numerous experiments involving administration of androgens to pregnant cows have so far failed to alter the gonads, although the external genitalia were masculinized (57). It has, therefore, become increasingly clear that the humoral theory alone is inadequate to explain the mode of origin of the cattle freemartin, and that cellular mechanisms may also be involved. For instance, cattle twins, including heterosexual ones, frequently have identical blood groups and represent red cell "chimeras." This finding suggests that an interchange of blood-forming cells between fetuses occurs *in utero* (71). Additional support for this view has come from the work of Anderson *et al.* (7) who have shown that identical (monozygous) twins in cattle could not be detected by reciprocal skin grafts, since even heterosexual twins sharing a common uterine blood supply were tolerant of each other's tissues in adult life. It can be inferred that transplantation antigens had been exchanged via the chorioallantoic anastomoses at a time when the fetus was immunologically tolerant, i.e., unable to distinguish between "self" and "nonself." Functional anastomoses are present between the allantoic vessels of cattle twins as early as day 30 of gestation, at a stage when germ cell migration normally occurs (70). Consequently, germ cell chimerism may be the cause of sterility in freemartins. This cellular theory of the origin of freemartins is not proven, as there is no direct evidence that the presence of XY germ cells in a genital ridge of XX cells will transform that ridge into a testis, although in Short's view (81) it is probable that the freemartin is masculinized by the secretion of its own gonads. Alternatively, Witschi (91) has suggested that the gonad of the freemartin may be transformed by a male inducer substance ("medullarin") passing from the male to the female co-twin. The sexual organogenesis of the freemartin has been described by Jost *et al.* (58). For a further discussion of the freemartin in cattle see Chapter 16.)

Freemartins, can, very rarely, occur in other domestic animals. Isolated cases have been reported in pigs, goats, and sheep (82). It has also been shown that, as in the cow, the sheep freemartin is a genetic female as well as a bone marrow chimera (42). The incidence of vascular anastomoses in sheep twin pregnancy is, however, only 5-10%, or far lower than in the cow, and that of red cell chimerism about 5% (30, 84). This fact probably accounts for the extreme rarity of freemartins in sheep.



It is thought that the avascular, necrotic tips of the chorionic sacs in the pig may discourage the formation of vascular anastomoses between one conceptus and its neighbor, and may therefore be important in controlling the incidence of freemartins in pigs. The avascular regions are present as early as the 27th day of gestation and may inhibit vascular anastomoses during the critical period immediately following histological sex differentiation (40).

## IX. Immunological Problems of Pregnancy

### A. THE FETUS AS A HOMOGRAFT

The fetus inherits transplantation antigens from both parents. Except, however, in inbred lines, the antigens inherited from the sire will be foreign to the dam and, to this extent, the fetus can be regarded as a homograft. One of the basic questions regarding pregnancy is why the mother nourishes this foreign tissue within her uterus and does not reject it as she would a skin graft with the same transplantation antigens.

To account for this biological "success" of the fetal homograft, several hypotheses have been advanced, namely, that (1) the fetus is antigenically immature, (2) the immunological reactivity of the mother is reduced during pregnancy, (3) a physical ("fibrinoid") barrier exists between mother and fetus, and (4) the uterus is an immunologically privileged site [cf. reviews by Billingham (15) and Anderson (8)]. None of these hypotheses, however, provides a wholly satisfactory explanation. It has been suggested by Amoroso and Perry (6) that the endocrine activity of the trophoblast itself may be a factor in its survival at implantation, particularly in the epitheliochorial placenta of the sow, mare, and goat in which no protective fibrinoid layer is deposited.

### B. PASSIVE IMMUNITY

Newborn mammals have limited powers of antibody production and during the postpartum period depend on passive immunity by antibodies of maternal origin. In the horse, pig, and ruminants the transmission of antibodies after birth occurs by means of the colostrum, and the intact antibodies are absorbed from the infant gut. In the dog there is some transmission of passive immunity before birth, but most of it is transferred through the colostrum after birth.

The separation of the fetal and maternal circulations is not absolute, and there is occasionally an escape of fetal erythrocytes into the maternal circulation. The mother becomes sensitized to these foreign antigens on the red cells and produces antibodies, the consequence of which is hemolytic disease of the newborn. In horses, when this occurs naturally, the foal is born healthy, but develops the disease after feeding on colostrum. Hemolytic disease also occurs in mules, when it is due to anti-donkey antibodies, and it has occurred in pigs when the mother has been immunized to fetal red cells by blood group antigens contained in a swine fever vaccine. A review of passive immunity is given by Brambell (22).

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# 13 Hormonal Mechanisms in Pregnancy and Parturition

Hubert R. Catchpole

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## I. Introduction

Pregnancy is defined as the time span between the implantation of a fertilized ovum in the uterus and the expulsion of the fetus and its associated membranes at term. Implantation may be a precise event, as when the ovum begins to erode the endometrium, as in rodents and carnivores, or more indefinite in time, as in the Equidae, when fetal and maternal tissues stay appositional and easily separable for a period of weeks. Parturition involves a prepartum interval during which contractions of the uterus are initiated and which is followed by the postpartum epoch when

the genital tract involutes and returns to a resting and eventually cyclic state. Pregnancy consumes a lengthy period in the economy of the individual mammal, lasting 3 or 4 weeks in laboratory rodents, 3-10 months in common domestic forms, and 2 years in the elephant. Exceptions are the hamster (15 days) and marsupials where very immature fetuses are born at 12.5 days and migrate to the mammary pouch where they are conserved for prolonged periods. For convenience of reference, selected gestation times are given (Table I) and the reader is referred to Kenneth's compilation (51) and to Asdell's valuable "Patterns of Mammalian Reproduction" (5).

The considerable changes occurring during pregnancy are, in general, mediated by hormones. A primary adaptation is the accommodation of the uterus to the constantly growing conceptus. During pregnancy, the new organism or organisms achieve a total weight which is a significant fraction of the mother's body weight: up to 33% in the mouse, 15% in the sow, and 10% in the thoroughbred. This presupposes adequate nutrition of the mother and uninterrupted transport and exchange of materials between mother and fetus across the placenta.

As the uterus increases in size to accommodate the fetuses, membranes, and fluid-filled sacs, some thinning of mucosal and muscular layers may occur, but there is an overall increase in total wet and dry weight in which

TABLE I  
Length of Gestation, Litter Size, and Result of Oophorectomy during Pregnancy\*

Species	Litter size	Length of gestation (days)	Aborts if oophorectomized before given day of gestation
Cat	4	63	50
Cow	1-2	277-290	Term
Dog	Multiple, mean 7.0	61	Term
Guinea pig	2-4	68	38
Goat	1-3	145-151	Term
Horse	1	330-345	150, 200
Monkey ( <i>Macaca mulatta</i> )	1	168	25
Man	1	280 $\pm$ 9.2	30-60
Mouse	Multiple, mean 6.0	19-20	Term
Pig (domestic)	8-12	112-115	Term
Rabbit (domestic)	Multiple, mean 8.0	31	Term
Rat	6-9	22	Term
Sheep (domestic)	1-2	144-152	55

\* Data compiled from several sources, reference (15).

both cellular and extracellular elements of endometrium and myometrium participate. While stretching may be the proximate stimulus for uterine growth, the fact that a sterile uterine horn also enlarges in pregnancy points to the mediation of hormones.

The mammary glands are also involved in growth and preparation during pregnancy. A complex of mammatropic factors carry the glands to the point of lactation in the immediate postpartum period.

The placenta as a unique organ of pregnancy poses questions in relation to both mother and fetus. The concept of a feto-placental unit has come to the fore with an appreciation of hormonal functions of the fetus, some acting in concert with those of the placenta. An interest in all facets of transplantation immunity has called attention further to the paradox of immunological tolerance of the "semi-foreign" fetus as a general and unsolved problem of pregnancy.

An earlier review (14) dealt with the subject of hormone excretion in the urine of pregnant domestic animals. While such studies led to valuable results, it was realized that urinary values, which are cumulative, reflect only imperfectly the active blood status of a hormone. The possibility of assaying minute amounts of steroid and protein hormones has led to a surge of studies on the quantitation of hormones in the blood of pregnancy which will be considered in the present chapter.

Other active areas since the last review (15) have included: the implications of cellular binding of hormones as a prelude to action; the role of the releasing factors; the significance of hormone transport proteins; the chemical nature and biological properties of protein gonadotropins and the significance of the prostaglandins. All are topics of active exploitation at the level of the laboratory rodents but many are scarcely scratched for the domestic forms. These animals are indeed more often the source than the target of their own prolific hormones.

## II. Maternal Endocrine Patterns

### A. HORMONE PATTERNS IN BLOOD DURING PREGNANCY AND PARTURITION

No discussion will be offered regarding methodology. Hormone values are generally given in nanograms/milliliter ( $10^{-9}$  gm/ml) or picograms/milliliter ( $10^{-12}$  gm/ml), and rarely in International Units (IU). Information which has been taken from the literature has many gaps and is sometimes mildly conflicting. The curves presented are mostly composite and to some extent impressionistic in attempting to convey major trends. It

is hoped that they can serve as a basis for future additions or corrections. The main hormones depicted are progesterone, estrogen, and LH. Results for other hormones are described if they bear elements of systematic treatment.

## 1. Cattle (Cow)

Cycle 21–22 days; pregnancy 277–300 days (Fig. 1).

a. **PROGESTERONE.** During the estrous cycle, progesterone values varied from  $0.44 \pm 0.17$  ng/ml at estrus to a peak of 6 to 7 ng/ml from days 9–16, then fell to baseline. During the first 14 days of pregnancy, values were the same as during 14 days postestrus. Cycling animals then showed a decline, while pregnant animals attained values at or somewhat above cycling maxima and maintained them for most of pregnancy (64, 78). Blood progesterone fell rapidly to 1 ng/ml on the day of calving, and still lower during lactational anestrus (1, 4, 30, 31, 45, 46, 73, 76).

b. **ESTROGENS.** The major estrogens are estradiol-17 $\alpha$ , estrone, and estradiol-17 $\beta$ , the first two comprising the bulk of active hormone. They were studied principally in early and late pregnancy, as related to implantation and parturition, respectively. Mated and pregnant cows showed values of less than 5 pg/ml from day 3 to 39, contrasted with animals which returned to estrus and showed somewhat higher values for 14 days and a major peak of 24 pg/ml at proestrus (45). Between the 140th and 245th day, values were reported below or about 100 pg/ml (31), although Robinson *et al.* (68, 69) found higher values at day 200 (1200 pg/ml) rising to 2500 pg/ml by day 250. All authors agree on a rise after day 250, which accelerated after 20 days prepartum and reached a maximum between the fifth and second prepartum days which was variously reported to be between 1500 and 10,000 pg/ml. Other estrogens are present at about one-tenth the level of estrone. A rapid decline occurs some 8 hours prepartum to minimal or nondetectable levels on the day following parturition (1, 4, 31, 45, 46, 67, 69, 76). Estrogen values (Fig. 1) are given conservatively.

c. **LUTEINIZING HORMONE, LH.** There are few systematic studies. The proestrus peak was 40 ng/ml (4). Ten Holstein heifers had less than 1 ng/ml in maternal blood serum at 120 days (53) and the same value was recorded at term for a mean of four cows (46). Judgment is reserved on findings in a single Zebu cow showing baseline values of 0.5–3.0 ng/ml after the third or fourth month which were interspersed with short



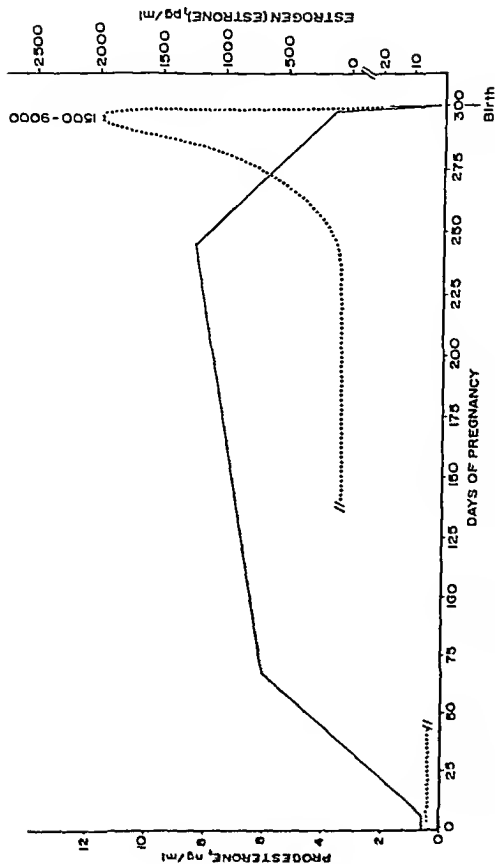


FIG. 1. Pregnancy blood levels of progesterone (solid line), and estrogen (dotted line) in the cow. Data interpreted from references (1, 4, 13, 30, 31, 45, 46, 53, 56, 67-69, 73, 76).

(3-day) bursts of hormone reaching peaks 100 to 2000 times the baseline values. After the 7th month, values of 1 to 4 ng/ml were the rule (13).

d. CORTICOSTEROIDS. Serum glucocorticoids near parturition (ten cows) averaged: day 26 to 1 day prepartum, 5 ng/ml; 12 hours prepartum, 10.3 ng/ml; parturition, 16.7 ng/ml; 12 hours postpartum, 5.1 ng/ml (76). A prepartum increase was also shown by Hoffman *et al.* (46).

e. ANDROGENS. Of interest in view of the possible relation to freemartinism, androgen assays are few and conflicting. Kiser (53) found no differences between testosterone and androstenedione in dams at 120 days carrying male or female fetuses, whereas Mongkonpunya (56) found a five- to tenfold difference for androsterone and a twofold difference for androstenedione in favor of dams bearing males, at 90, 180, and 250 days.

f. PROLACTIN. Starting from basal values of 50 ng/ml at 40 hours prepartum, prolactin levels rose to a mean peak of 320 ng/ml at 20 hours prepartum, then fell more slowly to baseline values by 30 hours postpartum (46). Similar findings were reported by Arije *et al.* (4).

## 2. Sheep (Ewe)

Cycle 16.4–17.5 days; pregnancy 144–152 days (Fig. 2).

a. PROGESTERONE. During a cycle, serum values varied from 0.12 ng/ml (equivalent to anestrus) to 2.0 ng/ml from day 10 to 14. After mating, pregnant and nonpregnant values did not differ till day 16, when values rose in the pregnant ewes to 2.5 ng/ml at day 50, 12–20 ng/ml at days 125–130, a plateau, and finally a steep fall to the day of lambing. Appreciable amounts were still present at the beginning of parturition, falling below 1 ng/ml at the end of the birth process (9, 36, 79, 81).

b. ESTROGENS. Few determinations are reported with the exception of the situation at term. Pregnancy levels were low (in comparison with the goat, for example), remaining less than 50 pg/ml for most of the period and rising to 100 pg/ml just 1 day before birth. On the day of parturition, mean levels rose to 400 pg/ml (one case showed over 2000 pg/ml), then fell to 50 pg/ml in less than 24 hours (81). Challis (18) first reported this estrogen peak in sheep and noted the terminal increase in the estrogen/progesterone ratio.

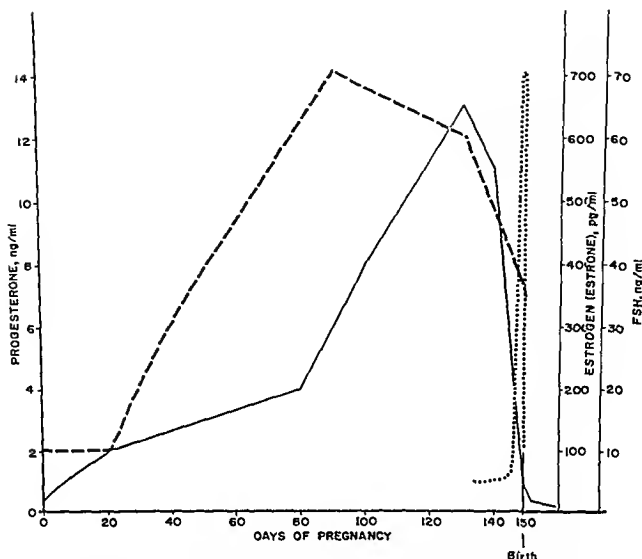


FIG. 2. Pregnancy blood levels of progesterone (solid line), estrogen (dotted line), and FSH (dashed line) in the ewe. Data interpreted from references (9, 18, 34, 36-38, 61, 71, 79, 81).

c. LUTEINIZING HORMONE, LH. Cycling ewes showed baseline levels of 2 to 3 ng/ml and peaks related to estrus of 30 to 200 ng/ml (37, 38, 71). Values dropped to less than 1 ng/ml in observations which continued to the twentieth day of pregnancy (61). At days 123-128, no maternal blood LH was detected (34).

d. CORTICOSTEROIDS. Plasma corticosteroids rose in control and ovariectomized pregnant ewes 72-78 hours prepartum (80). Values were: 11-19 ng/ml on days -8 to -3; 25-35 ng/ml on days -2 to +1. Over the same time interval, adrenalectomized sheep showed level values of 3 to 6 ng/ml.

e. FOLLICLE-STIMULATING HORMONE, FSH. The only figures found for FSH in pregnancy in a domestic form were those of Chamley *et al.*

(21). To the nearest integer, values were: day 42, 34 ng/ml; day 91, 72 ng/ml; day 126, 59 ng/ml; day 147 (parturition), 35 ng/ml.

f. **PROLACTIN.** Levels in the blood of pregnant ewes ranged between 20 and 80 ng/ml during the first 20 days. At 2 days prepartum a sharp rise began, reaching 400 ng/ml on the day of parturition with wide but smaller fluctuations for 8 days postpartum (29). Comparable results showed 20–40 ng/ml up to 80 days, an increase from 50 to 100 ng/ml within 10 days of parturition, and a terminal peak of 700 ng/ml representing a mean for seven ewes (50).

### 3. Pig (Sow)

Cycle 20–22 days; pregnancy 112–115 days (Fig. 3).

a. **PROGESTERONE.** Progesterone levels increased from 1 ng/ml on the day of estrus (mating) to a peak of 35.4 ng/ml on day 12 of pregnancy, then declined to 17.2 ng/ml on day 24 (41). In the final 20 days before birth, progesterone showed 8–14 ng/ml, with possibly a tendency to decrease in the last few days, and a fall to less than 1 ng/ml at birth (6).

b. **ESTROGENS.** Subsequent to the estrous peak of 40 pg/ml, and mating, estrogen remained around 20 pg/ml for the first 24 days of pregnancy (41). Between the 20th and 10th day prepartum, the level was about 100 pg/ml, peaked to 300 ng/ml at days –2 to –1, and fell to zero after delivery of all the piglets (6). At least 50% of the estrogen was estrone.

c. **LUTEINIZING HORMONE, LH.** Following an estrous peak of 4 ng/ml, LH dropped to values of 1 to 2 ng/ml for the first 20 days of pregnancy (41). Later values have not been found.

d. **CORTICOSTEROIDS.** The mean concentration during late pregnancy in sows was  $33 \pm 1.5$  ng/ml, with appreciable daily variations and no consistent elevation at term (6).

### 4. Goat (Doe)

Cycle 21 days, pregnancy 149 days (Fig. 4).

a. **PROGESTERONE.** Plasma progesterone was measured during pregnancy in the peripheral blood of sixteen common Norwegian goats before

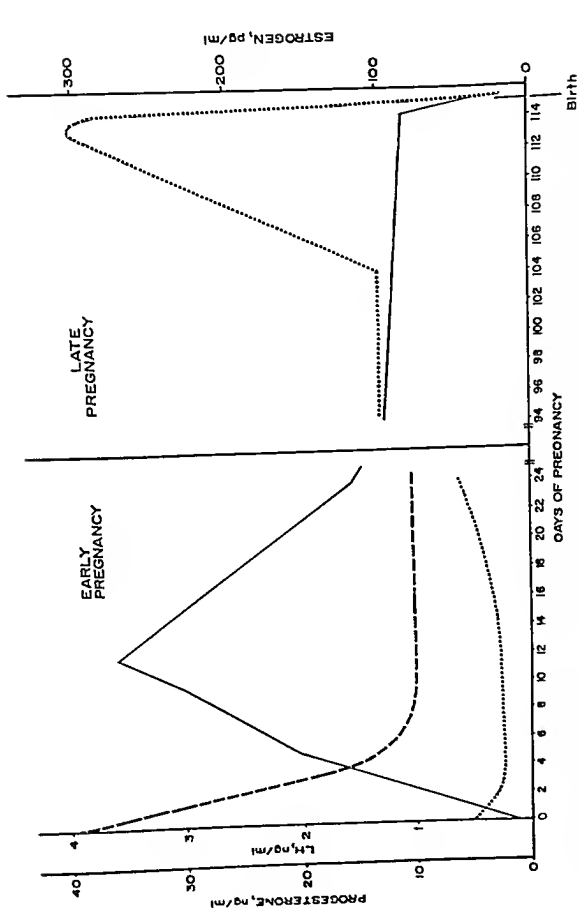


FIG 3 Pregnancy blood levels of progesterone (solid line), estrogen (dotted line), and LH (dashed line) in the sow. Data from references (6) and (41).

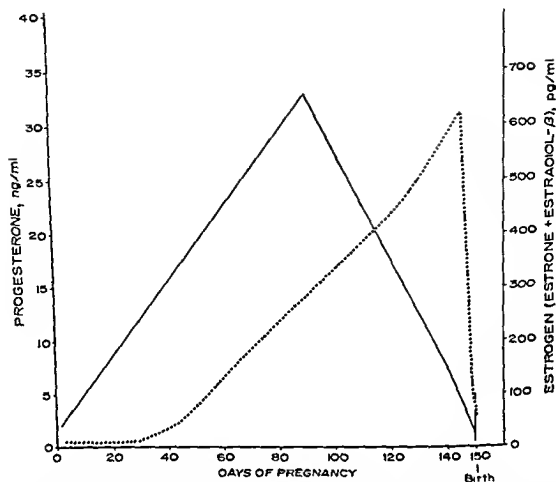


FIG. 4. Pregnancy blood levels of progesterone (solid line) and estrogen (dotted line) in the doe (goat). Data from references (11, 19).

and after extirpation of the ovaries and uterus (11). It was also studied in the ovarian vein. Levels rose gradually to 33 ng/ml by the 90th day and then fell to 7 ng/ml on the 140th day, 3 or 4 days before parturition. Ovarian blood levels were approximately 100-fold these amounts. In two does, sampled before and after parturition, plasma progesterone was found to decrease before birth and to remain low for at least 3 days after birth.

b. ESTROGENS. Estrogens were determined in the blood of fifteen goats, comprising four breeds, throughout pregnancy (19). Both estrone and estradiol-17 $\beta$  were present. Values recorded (to the nearest integer) were: days 0-30, 5 pg/ml; days 39-48,  $47 \pm 15$  pg/ml; days 79-88,  $272 \pm 35$  pg/ml; days 119-128,  $451 \pm 70$  pg/ml; days 139-148 (term),  $622 \pm 78$  pg/ml; days 1-10 postpartum, residual or undetectable amounts.

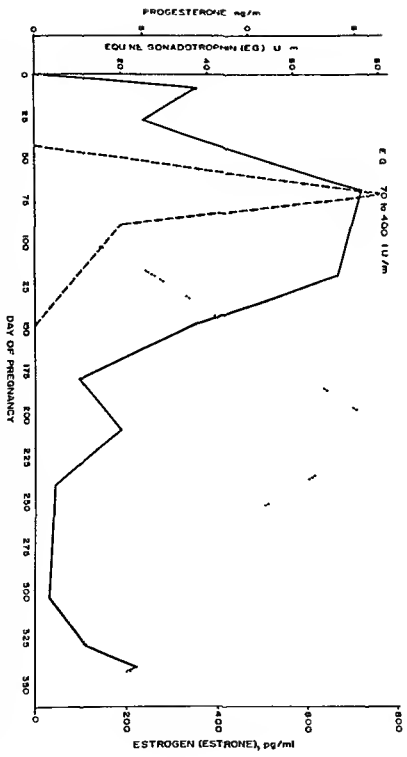


Fig. 5. Pregnancy blood levels of progesterone (solid line), estrogen (dotted line) and equine gonadotropin (dashed line) in the mare. Data largely from references (48, 60) with cross reference to reference (14).

tion beginning abruptly on the 42nd day and peaks of 50 to 100 IU/ml at 60–75 days. This schedule held for mustangs, thoroughbreds, Percherons, Welsh ponies, and Shetlands, with smaller breeds often tending to lower peak values (to 400 IU/ml). Mule pregnancies showed low titers, suggesting control by the fetal genotype. It is desirable that modern methods be applied to the situation prior to the onset of the hormone surge, also to titer both LH and PMSG, assuming these to be different hor-

## 6. Dog (Bitch)

Proestrus 5–12 days, estrus 5–12 days; pregnancy 65 days (Fig. 6)

Timing of events in the dog was considered best expressed as days from the LH peak. This is normally followed by ovulation in 12 to 24 hours. All reported determinations were done on pure-bred beagles.

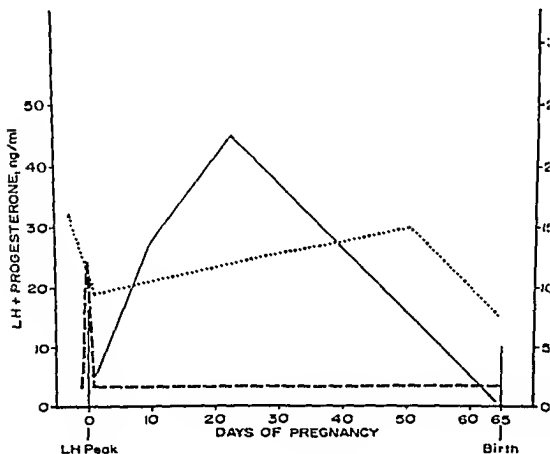


FIG. 6. Pregnancy blood levels of progesterone (solid line), estrogen (dotted line), and LH (dashed line) in the bitch. Data interpreted from references (26, 49,



appearance of the hormone. Less than 1 IU/ml was present on day 36 (not detected), by day 57, hormone peaked at 67 IU/ml then declined to less than 1 IU/ml by day 150. Early reported assays showed secretion beginning abruptly on the 42nd day and peaks of 50 to 100 IU at 60–75 days. This schedule held for mustangs, thoroughbreds, Percherons, Welsh ponies, and Shetlands, with smaller breeds often tending to high peak values (to 400 IU/ml). Mule pregnancies showed low titers (22), proving control by the fetal genotype. It is desirable that modern assay methods be applied to the situation prior to the onset of the hormone and also to titer both LH and PMSG, assuming these to be different hormones.

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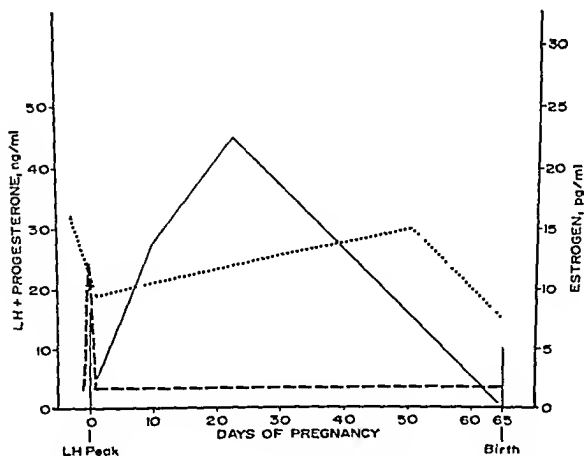


FIG. 6. Pregnancy blood levels of progesterone (solid line), estrogen (dotted line), and LH (dashed line) in the bitch. Data interpreted from references (26, 49, 75).

a. **PROGESTERONE.** Progesterone levels were below 0.5 ng/ml in proestrus but began to rise rapidly after the LH peak to maximum values of 25 to 30 ng/ml at 10 days, whether animals were mated or not. In unmated or sterile mated dogs, progesterone waned after 20 to 30 days to levels of less than 0.5 ng/ml after 80 days. Pregnant animals showed a significant increase at 20 to 25 days to a peak value of 40 to 50 ng/ml. Amounts then declined and were undetectable at parturition (75). Somewhat lower peak means were also reported (26, 49) as well as a longer maintenance of progesterone levels in pregnant versus nonpregnant (pseudopregnant) animals, and a definite decline during the last 2 days preceding birth.

b. **ESTROGENS.** Plasma estradiol-17 $\beta$  showed a maximum of  $17.2 \pm 3.8$  pg/ml in the first part of estrus, falling to a low of  $9.2 \pm 1.2$  in early pregnancy, rising to a fairly steady level of  $13.8 \pm 0.7$  pg/ml till day 50, and falling between days 51 and 60 to  $7.7 \pm 1.0$  pg/ml (49). This pattern did not differ materially from that of pseudopregnancy except for slightly higher levels in pregnancy. Higher overall values and a fall between the fourth prepartum day and birth were also reported (26).

c. **LUTEINIZING HORMONE, LH.** LH determinations were done in parallel with the above reported results in beagles. Pregnancy levels at about 2 to 4 ng/ml were maintained, similar to values in nonmated and sterile mated dogs. Only the estrus (preovulatory) peaks of 7.5 to 22 ng/ml break the monotony of these curves.

## 7. Rabbit (Doe)

Ovulation triggered by mating; pregnancy 30 days (Fig. 7).

a. **PROGESTERONE.** Progesterone and estradiol levels were studied in the peripheral blood of rabbits at 3-day intervals throughout pregnancy, augmenting previous results on limited periods and replacing earlier estimates of limited reliability (20). Mean values of progesterone rose from 5.3 ng/ml on day 3 to 17–19 ng/ml on days 12–15, gradually decreased to 6.1 ng/ml on day 30 (term), and to 1.9 ng/ml postpartum. A suggestion of a secondary peak at day 24 was not significantly different from values at day 21.

b. **ESTROGENS.** Estrone titers stayed in the range of 20 to 35 pg/ml throughout pregnancy with a suggestion of lower values at days 12 and 15. The mean values for estradiol-17 $\beta$  were nearly double those for es-

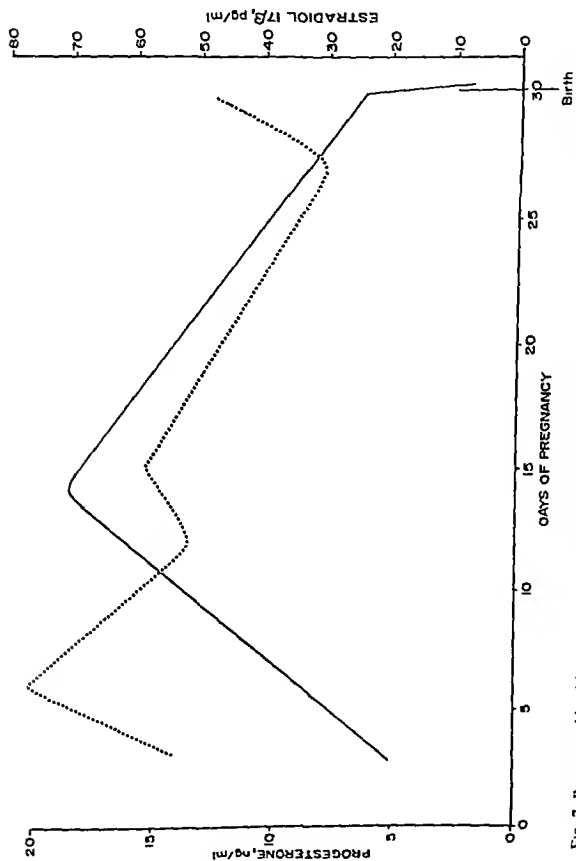


Fig. 7 Pregnancy blood levels of progesterone (solid line) and estradiol-17β (dotted line) in the doe (rabbit). Data from reference (20).

trone in the first 21 days and were more variable, showing peaks at day 6 (83.3 pg/ml), day 15 (59.0 pg/ml), and prepartum (48.4 pg/ml). In the final days there was no consistent predominance of estradiol-17 $\beta$  over estrone.

## B. THE MATERNAL OVARIES IN PREGNANCY

In the presence of fertilized and implanting ova, changes are initiated in the ovary which define its character for the remainder of gestation. Corpora lutea, which normally regress as a result of luteolytic actions, still not fully defined, become stabilized as corpora lutea of pregnancy. These bodies tend to be larger than corpora of the estrous cycle, although the individual cells are not greatly altered. These are typical steroid-secreting cells as defined histochemically and ultrastructurally. The growth of follicles which would normally signal the onset of another estrous cycle does not occur, and both ovulation and estrus are suppressed. This statement is subject to qualification: suppression of estrus may not be complete in early pregnancy. One to three ovulations in early pregnancy were reported to occur in Welsh ponies (2). Toward the end of pregnancy an estrogen-producing mechanism, at least partly ovarian, begins to reassert itself.

The question "how does the ovary recognize the fetus in the uterus" does not have a simple answer. In sheep, the signal is given by the blastocysts prior to implantation (58) and in cows an antiluteolytic effect of the conceptus is apparent by the 19th day, when progesterone levels of the pregnant and nonpregnant animals begin to diverge, and may be initiated several days earlier (72).

Ovariectomy at any point terminates pregnancy in the cow, goat, pig, rabbit, rat, and mouse, by resorption or abortion of embryos or fetuses (Table 1). Pregnancy can be maintained by the prompt and continuous administration of estrogen and progesterone in the proper amounts. This has been accomplished empirically in cows, gilts, and other forms. Ideally, administered hormones should attempt to maintain the correct values as determined by blood assays.

The ovary is dispensable in man after the 40th day, in the ewe after day 55, and in the mare after 150–200 days (two cases). Both in man and cow, earlier castration results in abortion and it is inferred that in these species an alternate source of estrogen and progesterone becomes available. In support of this, ovariectomy of pregnant sheep at 110, 122, and 135 days changed blood levels of progesterone very little, although the subsequent expected rise at the earlier times did not occur (36).

## 1. Role of Estrogens

The primary estrogen secreted by the ovary and possibly the placenta of all mammals is estradiol-17 $\beta$ . Following estrus and fertilization, blood levels of estrogens tend to fall in all species. Nevertheless estrogen continues to be secreted, and it is upon estrogen-primed tissues that the increasing amounts of progesterone are acting. Estrogen effects on the genital tract are: mitosis and multiplication of epithelial cells and glands; hypertrophy of smooth muscle cells and synthesis of contractile proteins; synthesis of cellular DNA and RNA; glycogen deposition in muscle cells of uterus and blood vessels; synthesis of glycoproteins and glycosaminoglycans of the uterus, cervix, and vagina. The estrogen-stimulated uterus takes up vital dyes and dye injected intravenously also accumulates at implantation sites in rodents on the 4th or 5th day (65) and in sheep on the 15th or 16th day (12). This reaction is commonly attributed to a local increase in vascular permeability. However, this conclusion can be criticized: dye accumulation may represent enhanced binding by altered extracellular matrix (16). Some of these actions of estrogen have relevance to the increasing demands being made on uterine metabolism. Others help to explain growth and physical adaptation to the conceptus. In most domestic species, elevated or rising concentrations of estrogen are characteristic of midpregnancy and some, like the sheep, cow, and sow display acute terminal rises.

Estrogen excretion in the urine of domestic animals (14) generally also increases as pregnancy advances, representing production which is partly ovarian but probably largely placental (or feto-placental), of hormone which is rapidly conjugated or metabolized to biologically less active derivatives. Estrogens and related steroids are found in the urine of pregnant sheep, pigs, goats, and cows, but man and the mare are the forms most prodigal in the production of active and inactive steroids. These are present in the blood and urine to the day of parturition, then abruptly disappear from the earliest voided urines.

## 2. Role of Progesterone

Blood assays reviewed above emphasize the primary role of progesterone in pregnancy. Its effect on the uterus is to add a qualitative change to the estrogen-prepared mucosa and to produce an enormous complication of the glandular pattern. The uterus becomes less hyperemic and the glands are coiled and inserted deeply in the mucosa. This progestational effect prepares the uterus for the reception of the fertilized ovum.

The role of progesterone for the remainder of pregnancy has been most eloquently summed up in the term "progesterone block" (28), namely, a dampening effect on the myometrium which inhibits contraction and which must be removed late in pregnancy to permit parturition to occur. Although suggestions were made as to the neuromuscular control mechanism, the phrase has remained essentially a descriptive one. Speculations concerning progesterone action on uterine muscle have run the gamut of every currently fashionable hypothesis.

Blood levels of progesterone universally tend to rise after mating. Absolute amounts vary with the species and are relatively lower in cow and ewe and higher in goat and sow. In some forms the amounts in pregnancy do not greatly exceed those in the corresponding sterile mated animal, e.g., the ferret (44) and the dog, which display a marked pseudopregnancy or metestrus. In most forms the pregnancy levels definitely exceed amounts found during the cycle, e.g., horse and pig (twofold), sheep (tenfold), and man (hundredfold). It is of interest that the highest levels of serum progesterone attained in domestic animals are much less than those recorded for laboratory rodents. Thus rats reach levels of 130 ng/ml on the 15th day of pregnancy (63) and guinea pigs 265 ng/ml at 35 to 40 days postcoitus (43). Size and metabolic factors may be involved, although man with 200 ng/ml in late pregnancy would be an apparent exception. Since the elephant is a domestic animal within the meaning of the Act, its low rate of progesterone synthesis by luteal tissue may not mean that the hormone is unnecessary as was suggested (74). A group of pregnant elephants (a staggering thought) would rather be expected to show low blood progesterones.

The situation in the mare is peculiar. Authors, including the present one, have stated that the primary corpus luteum of pregnancy is short-lived and that a multiple fresh crop takes over its function after the 6th to 8th week. This may be only partly true, since the primary corpus, which was tagged in experiments of Squires *et al.* (77), persisted morphologically and functionally and shared the common demise of all ovarian corpora lutea after the 160th to 180th day. Remnants of these bodies were seen at 220 days.

### 3. Role of Relaxin

This water-soluble peptide hormone is present in ovarian extracts and appears in the blood of pregnant rabbits, pigs, and cows. By immunoassay, relaxin levels were below 2 ng/ml during the first 100 days; rose to a mean of 12 ng/ml 3 days prepartum, then rapidly to 44 ng/ml at 30 hours and 146 ng/ml at 14 hours prepartum. Values fell to 42 ng/ml at 2 hours prepartum and to less than 1 ng/ml 1 day after birth (72b). Its

function is to widen the birth canal by softening or relaxing the fibro-cartilaginous ligament of the pubic symphysis, or, in cow and sheep by mobilizing the sacroiliac joints. Relaxin acts on connective tissues primarily by inducing an enzymatic depolymerization of such colloidal components of joints as the glycosaminoglycans and collagen. The hormone additionally acts on the uterus and cervix when given alone or with steroids (15) and acts synergistically with estrogen and progesterone to develop the mammary gland and suppress lactation (41a). Near term, prostaglandin  $F_{2\alpha}$ , which induces parturition, caused a marked rise in serum relaxin in sows when injected on day 112 of gestation (72a).

Purified pig relaxin is a polypeptide of molecular weight 6500 containing two chains (72c). The component A and B chains contain 22 and 30 residues, respectively, and the former has been sequenced (71a). Since the molecule contains no tyrosine or histidine, Sherwood *et al.* used<sup>1</sup> an iodinated polytyrosyl derivative to develop a radioassay method (72d).

## C. THE PITUITARY GLANDS OF PREGNANCY

The past 5 years have seen the characterization of two pituitary gonadotropins, FSH and LH, from beef, pork, sheep, and human pituitaries, and a description of their detailed chemistry and relationships with each other and with human chorionic gonadotropin and thyroid-stimulating hormone (TSH). FSH and LH are glycoproteins with a molecular weight of about 30,000 (40) comprised of two separable subunits, the hormones themselves representing dimers. With these materials in hand, it is hoped that functional studies of the pituitary will move off dead center. Little can be added to the meager information given previously (15) and no systematic studies of the gland in pregnant domestic species are presently available, either biochemical or histochemical.

### 1. Role in Maintenance of Pregnancy

Hypophysectomy of many common species before midpregnancy leads to fetal death and resorption. After this time varying degrees of failure are encountered. Rabbits and ferrets are totally intolerant; pregnant gilts operated upon at 70 to 90 days aborted within 60 hours (54), and goats operated upon at 38 to 120 days aborted in 3 to 9 days (25). Ewes hypophysectomized at 50 to 83 days went to term, but aborted if operated upon earlier. However, animals hypophysectomized at 10 days of pregnancy could be maintained by a combination of prolactin and LH, i.e., by known luteotropins. It is well known that the macaque monkey can be hypophysectomized on days 27 to 156, and delivers at the expected time of 168 days. The rule is supported that pregnant animals tolerate

hypophysectomy in inverse proportion to their dependence on ovarian estrogen and progesterone.

## 2. Pituitary Hormones in Blood of Pregnant Animals

Values of LH, FSH, and prolactin in the blood of pregnant animals have been given above. Quantitation of LH has yielded a limited amount of information which is far from startling. Ovulation is generally preceded by a burst of LH varying in amount with the species. Following mating, values settled at low but detectable levels in the cow (Fig. 1), sow (Fig. 3), and dog (Fig. 6). Ewes showed minimal to unobservable levels till 20 days and none later. A single report showed FSH to be present in the serum of pregnant ewes. Prolactin is also present during the first 20 days. A terminal rise in prolactin occurred at parturition in the ewe, goat, and cow.

The role of pituitary FSH in pregnancy is unknown. In the ewe, it appears to be curiously dissociated from LH, with which it presumably shares a releasing factor. The constant, if low, presence of LH in the blood of pregnancy in most species studied makes it an obvious candidate for the role of a luteotropin. The situation in sheep becomes easier to understand if the active entity in pregnancy is a luteotropic complex including both LH and prolactin.

## D. THE MAMMARY GLANDS OF PREGNANCY

Mammary gland development in an animal entering its first pregnancy depends on its hormonal history. The doe rabbit, which does not ovulate spontaneously, shows simple ductal growth and the rudimentary alveoli characteristic of estrogen stimulation while the dog with its prolonged luteal phase (pseudopregnancy) shows extensive mammary proliferation. Domestic species generally represent some intermediate variant. During pregnancy the mammary gland undergoes further stimulation and growth as a result of actions of gonadal and placental estrogens and progesterones.

Total mammary growth and lactation require a favorable environment of protein, carbohydrate, fat, water, and ionic metabolism and this involves the activity of a mammatropic complex which includes estrogens, progesterone, cortisol, thyroxine, and somatotropin (STH) as well as prolactin. Some of these hormones are directed to the mammary gland as a morphological unit; others to the production of an energy-rich secretion. It is reasonable to suppose that the appearance of enhanced amounts of prolactin at term in the cow, ewe, and goat signals the initiation of the latter process.



### III. Placental and Feto-Placental Endocrine Factors

The placenta has long been recognized as the "liver of the fetus" a massive metabolizing, synthesizing, storage, and transmitting organ. Placentas survive removal of the fetus in laboratory rodents, cats, and monkeys, and such persisting placentas are viable and exert an endocrine role and tend to be delivered at about the normal expected time. However, a normal "life-span" of the placenta has not been defined either histologically or biochemically.

#### A. STEROID HORMONES

Placental synthesis and interconversion of steroids is sufficiently documented by the appearance of some twenty-five or thirty different estrogens and progesterone metabolites in pregnancy urine. Most of these substances are biologically inert. Differing from the ovary, the placenta is an incomplete endocrine organ, lacking enzyme systems which can form steroids from acetate. In man, the placenta converts preformed steroid precursors such as maternal cholesterol to progesterone and fetal androgens, testicular or adrenal, to estrogens (70). Animals which abort after castration (rabbit, dog, sow, and goat) do not produce significant placental progesterone although estrogens are present in goat and sow placentas. In the cow, horse, and sheep, both hormones are produced by the placenta, although this does not save the cow from abortion when oophorectomized. It is apparent that the placenta must attain a certain maturity and mass before its functional contribution becomes significant.

#### B. PROTEIN AND POLYPEPTIDE HORMONES

The placental gonadotropin of man (HCG) has a subunit in common with pituitary LH and FSH of domestic animals and thus shares their molecular phylogeny. Its function as a luteotropic hormone seems certain.

Equine gonadotropin (PMSG) has now been removed from its anomalous position as a maternal uterine hormone and restored to its status as a true fetal product secreted by allantochorionic cells which become detached and permanently affixed to the uterine mucosa where they develop as specialized endometrial cups (3, 23). Although it was obtained early in highly purified form, PMSG has not received an intensive chemical scrutiny. From its molecular weight of 68,000 (62), it is probably a tetramer, and is biologically unique by reason of its retention by the kidney, its long half-life in the circulation, and its combination of LH and FSH properties. The conclusion that it is responsible for ovulation and

corpus luteum formation in early pregnancy in the mare was criticized (8) on the reasonable grounds that ovarian activity precedes its appearance in the serum. However, there is the possibility of a local action of the hormone via uterine vein to ovarian artery, before its appearance in the general circulation. In later pregnancy it seems certain that its function is luteotropic.

### C. PLACENTAL LACTOGENS AND LUTEOTROPINS

Interest has continued to center on a polypeptide hormone, HPL (human placental lactogen), having molecular homologies with growth hormone and prolactin. Since this substance is also luteotropic in the rat, it should be included in both luteotropic and mammatropic complexes of pregnancy. A rat placental luteotropin (RPL) with a double action on the life-span of the corpus luteum and on the mammary gland has been isolated (35). Whether materials of this kind are related to the luteotropic activity of blastocysts in early pregnancy is unclear (58, 72). Possibly the better chemical definition of lactogenic and gonadotropic hormones in the placentas of domestic species which, while lacking overt hormonal activities, could show immunochemical affinities with existing hormones. Some mysterious absences, as well as presences, of hormones in the placenta might be explained by such studies.

## IV. Fetal Endocrine Functions

The primordia of the endocrine glands are laid down early in intra-uterine life. The glands differentiate so that by the time of delivery they usually possess a histological type of recognizably adult pattern. A systematic study of fetal endocrines would seek to determine (1) when the gland becomes hormonally competent, (2) the reactivity of fetal endocrines with each other, (3) the response of fetal endocrines to maternal hormones which cross the placenta, and (4) the contribution of fetal secretions to the placenta and maternal organism. Only a few aspects of this gigantic program have been worked on or can be considered here. Some were previously reviewed (15).

### A. FETAL GONADS

The genetically determined gonads of both sexes develop in a maternal milieu and it has been thought that the male gonads, through secretion of testosterone or other male hormone, may contribute to the development of the male accessories. More controversial is the possibility of male hormones, through cross-circulation in a female co-twin, modifying gonadal development leading to the syndrome of freemartinism in cattle, sheep,

and pigs. Evidence of early synthesis of androgens have been provided for fetal pig testes as early as a crown-rump length of 1.5 cm (57), in cattle fetal testes from 90 to 260 days (52, 56), and in sheep fetuses between 30 and 70 days (7).

The fetal gonads of the horse display a remarkable proliferation of interstitial cells reaching a maximum at 6.5 to 8 months and degenerating before term. Aristotle has the priority on this observation, and the gonads have been studied histologically (24) and ultrastructurally (39). These gonads are not particularly rich in active steroid hormones but they may supply  $C_{19}$  precursors to the placenta. Modern hormonal studies are needed. The results of surgical removal of the fetal gonads tend to support the idea that they serve as the ultimate source of maternal blood and urinary estrogens in the mare (66). No new evidence has come to light to show whether equine gonadotropin initiates this interstitial cell proliferation or if another mechanism should be sought.

### B. FETAL HYPOPHYSIS

A potential activity of the fetal bovine pituitary at 120 days was shown by instilling gonadotropin-releasing hormone (GnRH) into six male and four female fetuses via arterial cannula, and the prompt appearance of LH in the fetal serum (53). In the male fetuses, serum testosterone increased, indicating a gonadal response as well. In rabbits, hemidecapitation at 22 days to remove the hypophysis significantly reduced thyroid and adrenal weights at 29 days (10).

### C. FETAL ADRENALS

Sheep bearing adrenalectomized lambs do not deliver spontaneously while stimulation of the fetal adrenals with ACTH or administration of cortisol to the fetus causes premature delivery. Glucocorticoids were considered responsible for initiation of parturition in this species (55). Also in fetal rabbits, cortisol levels rise in the last hour of gestation (59).

### D. COMMENT

The above fetal endocrine systems, as well as others such as the thyroid-thyrotropin system, become functionally active surprisingly early, begin to modulate processes within the fetus, and may affect events in the mother including parturition in the ewe. In the mare, the fetal genotype determines the reaction between allantochoial cells and endometrium and the amount of equine gonadotropin secreted. At the 40th day of pregnancy, large amounts of the glycoprotein hormone PMSG are associated with the stallion to mare allograft, and small amounts with the Jack donkey to mare superallograft, a piece of information which, at present, defies interpretation.

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## V. Hormonal Aspects of Parturition

Observations of the domestic species have shown that the age and parity of the mother, and the sex, size, normality or abnormality, and genotype of the fetus may all affect gestation length (47). The last was stressed by Holm, and is illustrated by the phenomenon of prolonged gestation in common dairy breeds and by the gestation lengths of the horse (340 days), hinney (350 days), mule (355 days), and donkey (365 days) (15). Experiments already quoted on the persistence of the placenta after fetal removal do not negate the role of the fetus, but rather emphasize that important elements of the parturition process are located at the uterine-placental locus. These influences are strongly hormonal in nature and revolve around intricate relationships between estrogens, progesterone, adrenal steroids, oxytocin, relaxin, and prostaglandins.

### A. ESTROGENS AND PROGESTERONE

These hormones are always present in the uterine milieu. It is known that their relative proportion is usually crucial in determining a uterine response, e.g., the varying capacity of different absolute amounts and proportions in preventing uterine breakdown and menstruation contingent on castration in the macaque monkey (17). If pregnancy represents a situation of relative progesterone excess, there is evidence in laboratory rodents and in the rabbit, pig, sheep, and cow for one or more of the following: (1) a terminal fall in blood progesterone, (2) prolongation of pregnancy by progesterone injection, (3) termination of pregnancy by ovariectomy unless progesterone is supplied. Rising amounts of estrogens characterize mid- and later pregnancy in man, mare, cow, sheep, goat, and pig, with terminal peaking in the case of the last four. Estrogen is an anabolic hormone for the genital tract and mammary gland and this may represent its function during most of pregnancy, exclusive of implantation. Data on blood hormone levels support, in general, an increase in the estrogen/progesterone ratio as a major correlate to renewed activity of the myometrium as the conclusion of gestation approaches.

### B. OTHER FACTORS

The role of oxytocin has been enigmatic since its discovery by Dale in 1909. In the rabbit, endogenous oxytocin was shown to initiate the birth process (27), and in the goat oxytocin increased during the second stage of labor. However, an increase in the estrogen/progesterone ratio would be expected to sensitize the uterus to oxytocin whether its absolute secretion increased or not. In goat, ewe, and cow, while not essential for

the induction of labor, oxytocin may be released in response to distension stimuli to the vagina and vulva, and facilitate parturition through its contractile action on the uterus

The mechanism of involvement of the fetal adrenal glands in sheep and rabbits, where it is being most intensively studied, is not clear. Fetal adrenalectomy does not prevent the induction of labor in the mother with glucocorticoids or affect the surge in estrogen levels at term in the sheep (33), nor do fetal plasma corticosteroids cross to the maternal plasma in late pregnancy in this species (80)

In several species an increased production of prostaglandins by the term uterus is reported. Mechanisms whereby this could potentiate the natural expulsion of the uterine contents are legion and the whole situation bristles with degrees of freedom involving most of the above variables (32)

### C General Comment

A unique mechanism for parturition has not been elucidated. Parturition possibly more than other aspects of reproduction has suffered from all-or-none positions. Insofar as the process is a hormonal one, means are now in hand, by the measurement of blood variables, to begin to disentangle the various effectors that appear to be necessary and active as the new organism achieves its independent existence.

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# 14 Mammary Gland Development and Lactation

R. L. Baldwin and Teresa Plucinski

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## I. Introduction

The preparation of a general, concise treatment of mammary gland morphogenesis, growth, and the initiation and maintenance of lactation requires extensive reliance upon references to previous treatments of the subject. These studies provide guidelines for new development of knowledge in this area and furnish information for further detailed treatments of mammary growth and lactation. A number of comprehensive treat-

ments of lactation have been published (13, 21, 33, 36, 55, 58, 69). These should be consulted for detailed aspects regarding mammary anatomy and development (13, 33, 58, 69), the initiation and maintenance of lactation (13, 33, 36, 55, 58), hormonal control mechanisms (13, 21, 36, 55), and mammary metabolism (21, 36). Emphasis in this chapter is placed upon summarizing information on the mammary gland and lactation, as well as critical evaluation of recent literature in selected areas of current research emphasis.

## II. Anatomy, Morphogenesis, and Development of the Mammary Gland

### A. ANATOMY

Turner, (69) has considered both gross and microscopic anatomy of the mammary glands of cattle, other hoofed animals, and marine mammals. Others have considered the anatomy of glands of a number of additional species (11, 22, 27, 28, 33, 51, 59). Several species differences in gross anatomy are apparent as exemplified in Figs. 1 and 2. In many species, major ducts, which provide for milk removal from the secretory

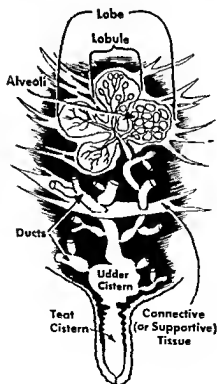


FIG 1. Schematic drawing of a cow's udder, showing the major structures of the teat, the gland cistern, the connective stroma, and lobuloalveolar structure (from Turner, 70).

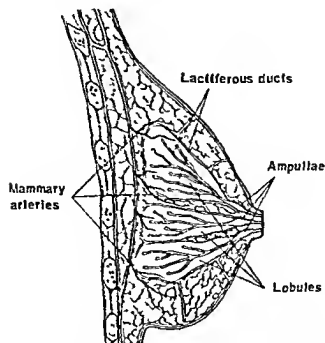


FIG. 2. Schematic drawing of human breast, showing ducts leading to nipple from ampulla and the intermingling of lobuloalveolar elements with adipose tissue [from Baldwin (15) "Animal Agriculture: The Biology of Domestic Animals and Their Use by Man" (H. H. Cole and Magnar Ronning, eds.). W. H. Freeman, San Francisco, California. Copyright © 1974.]

parenchyma, terminate in the nipple (Fig. 2), while in ruminant species, these terminate in a gland and teat cistern (Fig. 1). At the microscopic level there is similarity among species. The alveolus is the basic secretory element of the mammary glands in all except very primitive mammals (Fig. 3). The alveoli are small vesicles or sacs made up of a single layer of secretory epithelial cells which surround the lumen. The alveoli are surrounded by a basement membrane, fine networks of capillaries, and myoepithelial cells. Alveoli are arranged in lobules (Fig. 2) and are drained by intralobular ducts. These ducts connect with interlobular ducts which join larger ducts and provide a route for milk removal from the gland. Lobules are surrounded by connective tissue and are arranged in lobes, also surrounded by connective tissue elements. Lymphatic capillaries are found in the interlobular connective tissue but not in the intralobular area. Extensive light and electron microscopic studies indicate only minor interspecific differences in mammary secretory epithelial cell structure (28). Fully developed mammary secretory cells have very prominent nuclei, mitochondrial, and Golgi structures and an extensive rough endoplasmic reticulum. Early speculations regarding mechanisms of milk secretion by alveolar cells were reviewed by Turner (69). The general conclusion was that although secretory cells undergo considerable destruction as evidenced by the presence of enzymes and subcellular particles in milk, the mechanism of secretion must be conservative or meso-

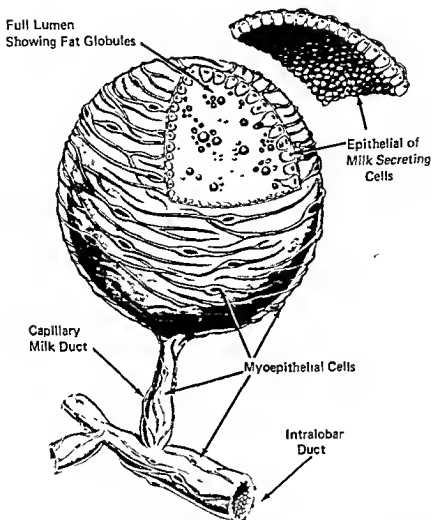


FIG. 3. A schematic drawing of an alveolus, showing how the mammary secretory cells are arranged to form a saclike structure, how the lumen, into which milk is secreted by the secretory cells, is connected to an intralobar duct, the close relationship between blood capillaries and the alveolus, and the myoepithelial cells which contract to expel milk from the alveolus (from Turner, 70).

crine in nature. It was also considered that secretion during milk removal from the gland might be partially holocrine in type resulting in cell decapitation. Recent studies have clarified further, though not completely, the mechanisms of secretion of milk components. Electron microscopic and biochemical observations indicate that the membrane which surrounds milk fat globules is formed from the plasma membrane (32, 57). Also, subcellular particles are often found in close association with milk fat globules isolated from milk. These observations support earlier views that fat secretion involves a form of reverse pinocytosis in which the portion of

cell membrane surrounding fat droplets is lost when the plasma membrane envelopes the fat globule as it is secreted. The mechanism is not entirely conservative since cytoplasmic elements are secreted along with the fat droplet. Current data (20, 37) indicate that casein micelles and lactose are formed in the Golgi apparatus of secretory cells. These might be secreted either via Golgi tubules which are contiguous with the plasma membrane or via vesicles which are released by the Golgi, drift toward the plasma membrane and, in the process of joining the plasma membrane, release lactose and casein into the alveolar lumen (10, 32, 57). Both of these possibilities imply that secretory cell plasma membranes are formed from Golgi membranes (32).

## B. MORPHOGENESIS

The basic sequences of events during morphogenesis of the mammary glands are similar in most species (1, 53, 69). Two milk lines become apparent on the abdomens of very early fetuses. Epidermal thickenings are soon formed at points along the milk lines which correspond in placement to the points at which mammary glands will be formed in the particular species examined. In the case of the bovine fetus, for example, four epidermal thickenings are formed on the milk lines in the inguinal region between the hindlimbs. The epidermal thickenings are called "mammary buds." The mammary buds themselves do not differentiate to form mammary structures, but rather serve as a focal point for the differentiation of dermis, epidermis, and mesenchyme. Early in development, the mammary buds sink into the mesenchyme and a condensation of mesenchymal cells takes place around the bud causing the appearance of embryonic teat hillocks. Also at this stage, ectodermal cells overlying the bud proliferate to form a neck of epidermis connecting the mammary buds to the epidermis at the apex of the teat hillock. In subsequent stages, the epidermal neck increases in size and forms a funnel-shaped cone at the base of which an epithelial cord or primary sprout extending the length of the fetal teat develops. The primary sprout canalizes starting at the apex of the teat during the 19-cm stage of the bovine such that when the fetus reaches 30 cm in length, structures corresponding to the streak canal and teat cistern are clearly defined. These are composed of a basement membrane and a double-layered lining of epithelial cells. At the base of the teat a clearly defined structure corresponding to the gland cistern, formed from the primary sprout during canalization and lined with a double layer of epithelial cells, is evident. Secondary and tertiary sprouts representing the beginning of the duct system of the gland leave the cistern and penetrate the surrounding mesenchyme in various directions. During later stages of

fetal development, the mesenchyme differentiates to form the connective tissue which will support the fully developed mammary gland, the connective glandular stroma which will ultimately surround the lobuloalveolar structures of the mammary gland, and adipose tissue which surrounds the glandular elements and comprises the bulk of the udder at birth.

The duct system is still rudimentary at birth and is confined to a very small area surrounding the gland cistern. The teats are well-formed with the exception that the sphincter muscle surrounding the streak canal at the apex of the teat and the smooth muscle cells surrounding that teat cistern are not clearly evident. The connective stroma and the vascular and lymphatic systems are reasonably well developed.

Data summarized by Raynaud (54) and Anderson (1) indicate that hormonal influences are not necessary for early mammary development in the female fetus, although insulin and growth hormones stimulate development of mammary buds cultured *in vitro*. The primary hormonal effect upon fetal mammary development occurs in the male as a result of the secretion of androgens by the developing testes. The androgens inhibit teat growth and cause detachment of the primary mammary sprout from the external epidermis. The steroid hormones, especially estrogen, although not required for development, can produce alterations in the pattern of development and when injected in large doses can produce abnormal development.

## C. PREPUBERTAL DEVELOPMENT

The development of the mammary glands from birth to puberty is characterized by generalized growth and maturation of elements not clearly defined at birth, such as the teat sphincter and smooth muscle fibers. A number of techniques have been employed to investigate changes which occur during this period. Matthews *et al.* (43) and Swett *et al.* (65) used gross morphological criteria such as changes in the weights, dimensions, and capacities of udders of heifers to characterize growth and development. Several investigators (11, 22, 59) employed histological techniques for quantitative measurement of development and degree of branching of the mammary duct system.

Udder weight in calves was highly correlated with age (43). Udder size as assessed by palpation of calves between 3 and 5 months of age was somewhat related to subsequent lactation performance, an observation which led to the proposal that these measurements might be employed to cull calves of low production potential at an early age (65). However, the usefulness of size and weight criteria for predicting future lactational performance has been seriously questioned (49, 69) on the grounds that

individual variance is very large and that these criteria do not distinguish between development of the mammary parenchyma and growth of associated adipose tissue. Criteria based upon measurement of increases in gland capacity or internal volume determined by injection of fluids appear to be useful; these have not been employed extensively to assess development, however, because of the difficult and unphysiological nature of the measurement. A steady increase in udder capacity or internal volume, presumably reflecting growth of the gland cistern and duct proliferation, has been reported for a number of species (43, 49, 65).

Measurements of mammary duct growth, degree of branching, and duct area in whole-gland mounts have been employed to characterize prepubertal mammary gland development in mice and rats (22, 59). Mammary gland growth was isometric with respect to the rate of growth of the body as a whole for a period after birth, but became allometric (faster than the overall growth rate) several weeks prior to the onset of estrous cycles. In ovariectomized females and normal and castrate males, mammary gland growth rates were isometric throughout this period. The whole-gland mount technique is difficult to employ for the study of rats and mice after puberty because the glands start to develop in three dimensions at this time. The technique assumes a two-dimensional model. For this reason, the technique cannot be employed to study prepubertal development in species such as guinea pigs and goats, whose glands have a three-dimensional structure at birth. Techniques for the study of pre- and postpubertal growth in mammary glands having a three-dimensional structure have been developed (49). These techniques involve tedious measurements of percentage of gland parenchyma and of average, cross-sectional areas of serial or sample sections of the gland. Results obtained with these techniques confirm the observation that mammary growth is isometric early in life and becomes allometric prior to puberty as a result of prepubertal ovarian activity (11).

#### D. POSTPUBERTAL DEVELOPMENT

Although allometric growth of the glandular epithelium commences prior to the onset of puberty, puberty is generally considered to represent the time during which a rapid acceleration of the mammary development occurs. At the onset of puberty the gland cistern and the large ducts which lead from it are composed of a double layer of epithelial cells bordered by a slightly developed basement membrane surrounded by layers of connective tissue. The smaller ducts are composed of a single layer of epithelial cells surrounded by thin, delicate layers of connective tissue. Following puberty, the smaller ducts proliferate very quickly while estrogen

levels are high and regress slowly during other phases of the estrous cycle. In each cycle, more ducts are formed during the period of proliferation than are lost during the period of regression with the net result that a highly branched matrix of ducts is slowly developed. This matrix represents the beginning of the development of true lobular structures. The proliferation of ducts and ductules is accompanied by or, in the human, preceded by development of the connective tissue stroma, growth of adipose tissue, and further development of the myoepithelium and the vascular and lymphatic systems. It is at this stage that species differences arise due, in part, to differences in estrous cycles and, in part, to inherent species differences in the response of glandular elements to the ovarian hormones. In the latter case, it has been noted that in cows, goats, and rodents (44, 69) adipose tissue growth is rapid and development of the connective tissue stroma lags behind duct growth. In the human, adipose tissue growth is slower and the connective stroma becomes highly developed. Mayer and Klein (44) noted that, in general, species which have short estrous cycles with a very short luteal phase exhibit, primarily, duct growth, while in species such as the dog where the cyclic corpus luteum is very long-lived, duct growth is accompanied by considerable lobuloalveolar development. Hence, estrogen is considered to be largely responsible, either directly or indirectly, for duct growth; lobuloalveolar development is considered to be dependent upon progesterone.

#### E. DEVELOPMENT DURING PREGNANCY AND EARLY LACTATION

The mammary gland attains its maximum development during pregnancy or immediately thereafter. The development which occurs during this period is dependent upon estrogen, progesterone, and pituitary hormones. The role of pituitary secretions in development will be discussed in detail in Sections III, B and C.

Numerous early studies, summarized in a comprehensive review by Meites (45), were undertaken with the goal of developing techniques for the artificial initiation of lactation. These studies were based on the premises that full mammary development occurs during pregnancy as a result of maintenance of an appropriate balance between progesterone and estrogen secretion and that simulation of pregnancy by administration of progesterone and estrogen to sterile cows leading to artificially induced lactation would be of benefit to the dairy industry. The most promising experimental techniques were based on administration of estrogen-progesterone combinations for a prolonged period to induce udder growth followed by administration of a "triggering" dose of estrogen and initiation of milking. In general, although estrogen-progesterone combinations have



been found which induce good lobuloalveolar development in several species (1, 8, 64); milk yields after artificial induction of lactation have been variable and below normal (45, 68). Because of the time and expense required for artificial induction of lactation by simulation of pregnancy and low milk yields during induced lactations, further investigation utilizing this approach was not deemed practical (45) and research on induced lactation was limited for a number of years. Recently, however, Smith and Schanbacher (61) reported that injections of estradiol-17 $\beta$  (0.1 mg/kg body weight) and progesterone (0.25 mg/kg body weight) for 7 days will initiate lactation in 60% of infertile cows. Milk yields in lactations thus induced were promising. These observations have renewed interest in the possibility of obtaining practical benefit by inducing lactation in infertile cows.

### III. Hormonal Requirements for Mammary Gland Development and Lactation

#### A. METHODS OF EVALUATION

##### 1. Criteria of Development

Several of the morphological and histological techniques that have been employed to characterize and quantitate mammary gland development were discussed in previous sections on development during morphogenesis, puberty, and early pregnancy. Many of these techniques have also been employed in studies of hormonal effects upon mammary tissue and in the study of changes occurring in mammary glands during late pregnancy and lactation. However, their usefulness during these periods is impaired by a number of factors. The difficulty in assessing development in glands possessing a three-dimensional structure was referred to in Section II,C. Other factors which affect the interpretation and usefulness of histological methods include distention of glandular structures by colostrum secretions during late pregnancy and accumulation of fluids in late pregnancy and early lactation. Differences in amounts of accumulated fluids and secretions must be corrected when measurements of cross-sectional areas and percentages of parenchyma in selected or sequential gland sections are used to assess growth and development. Several histological techniques which avoid these difficulties have yielded considerable information concerning gland development in late pregnancy and lactation. One of these uses numbers of cell divisions as a criterion of continuing growth and development. Numbers of cell divisions are usually determined by estimating

frequencies of mitosis directly, by estimating frequencies of mitotic events in glands after treatment with colchicine, or by estimating numbers of radioactive nuclei present after administration of tritiated thymidine (25, 31, 52, 63, 67). This latter technique provides a basis of assessing the types of cells being formed (67). Histological techniques have been extensively used to evaluate hormone effects upon lobuloalveolar development and milk formation in *in vitro* and *in vivo* systems. In the latter case, amounts of milk in the alveolar lumen and amounts of milk components in secretory cells are scored in a semiquantitative fashion to evaluate magnitudes of hormonal effects. Electron microscopy has been used extensively in recent years to evaluate hormonal effects upon ultrastructure (28, 66):

A number of biochemical techniques has been employed to study changes in numbers and types of cells in the mammary glands during pregnancy, lactation, and involution. Perhaps the most prominent index employed to study changes in cell numbers has been DNA content. Use of this index of cellularity implies acceptance of the assumptions that DNA content per nucleus remains constant throughout various stages and that no changes in average numbers of nuclei per cell occur. It is generally assumed (1) that the amount of DNA per nucleus remains constant throughout pregnancy and lactation in rats. However, Simpson and Schmidt (60) presented data in conflict with this assumption. Mayer and Klein (44) presented arguments indicating that numbers of multinucleate cells increase during late pregnancy and lactation. These observations indicate that mammary DNA data must be interpreted with care. However, measurement of total DNA is the most useful and convenient method available for assessing changes in mammary cellularity.

Numerous types of measurements of mammary metabolic activity have been used extensively as criteria for development. Included in these are estimates of oxygen uptake, rates of milk component synthesis, rates of oxidation of specific substrates by gland explants, slices or isolated cells incubated *in vitro*, and determinations of RNA and enzyme levels. Metabolic and enzyme data must always be interpreted with caution since these usually reflect metabolic capacity or potential and not, necessarily, *in vivo* activity (7).

## 2. *In Vivo* Techniques

Studies of the hormonal requirements for gland development and the initiation of lactation in intact animals have contributed significantly to the development of knowledge of hormonal relationships in mammary growth and lactation. Two types of studies can be considered. First, hor-

mones can be administered to intact animals and effects upon the gland evaluated. Second, blood hormone levels can be monitored during periods of mammary development such as puberty, pregnancy, and lactation and possible relationships between hormone changes and development can be evaluated. In the former case, interpretation is very difficult because the hormone treatments are usually imposed upon ill-defined or individually variable bases and injected hormones can affect mammary development indirectly as well as directly. Estrogens, for example, can alter ovarian function and prolactin secretion in addition to possibly stimulating duct growth and increasing the responsiveness of the mammary gland to prolactin (46). Confounding features in the interpretation of blood hormone data are that causal relationships between hormones and responses must be established and that tissue sensitivity to a hormone can vary with physiological state. In order to avoid secondary endocrine effects that can arise as a result of administration of hormones, various workers have preferred to use hypophysectomized, ovariectomized, and/or adrenalectomized animals to study hormone requirements for mammary gland development and the initiation and maintenance of lactation. Jacobsohn (30) emphasized that judicious evaluation of the effects of these various endocrinectomies upon the general physiological status of animals must be exercised. In cases where replacement therapies are employed, one must distinguish between "permissive" hormonal effects arising from improvement of physiological status and direct hormonal effects on the tissue. Despite the fact that it is sometimes difficult to distinguish direct from indirect hormonal effects, evaluations of hormone actions *in vivo* are essential.

### 3. *In Vitro* Techniques

In recent years, tremendous progress has been made in assessing the specific hormonal requirements for mammary gland growth and differentiation through the use of *in vitro* gland explant and cell culture techniques (66). The clear advantages of these techniques are rigorous control and characterization of experimental conditions and specific manipulation of the environment. The primary difficulties or limitations encountered appear to arise from the small amounts of tissue available for study from explants and rapid losses of secretory activities in explants and cells from lactating animals (19). These limitations make results difficult to interpret. Of particular concern is relating mechanisms established *in vitro* to the *in vivo* situation. Hormone actions *in vitro* leading to increased rates of milk synthesis, which are only 1 or 2% of rates observed in lactating tissue *in vivo*, may or may not be quantitatively important *in vivo*.

## B. DEVELOPMENT DURING PREGNANCY

Several aspects of mammary gland development during puberty and pregnancy were discussed above. It was implied that estrogen and progesterone are the primary hormones required for development in intact animals during this period, that estrogen stimulated duct growth, and that progesterone regulated lobuloalveolar development. In general, these implications are correct, but species differences, the role(s) of hormones from the anterior pituitary, and interactions between ovarian and pituitary hormones must also be considered.

Folley (23) considered species differences in response to ovarian hormones as representing three broad categories. Species in the first category include rats, mice, rabbits, and cats, which are described as exhibiting only duct growth when physiological doses of estrogen are administered. In these species, lobuloalveolar growth occurs only when progesterone is administered. The second category includes guinea pigs, goats, and cows, species which require both estrogen and progesterone for normal duct development. Some lobuloalveolar development occurs in these species when estrogens alone are administered. The bitch has been placed in a third category because little or no mammary development occurs when estrogen alone is administered. These species discrepancies may be due to differences in the actions of estrogens on glandular tissues, differences in the effects of estrogens on other tissues, including the ovaries and the anterior pituitary, differences in endogenous secretion of progesterone, differences in synergistic relationships between steroid hormones and prolactin and growth hormone, or, as is most likely, a combination of these.

The literature concerning the role of the anterior pituitary in mammary development during pregnancy is quite extensive (1, 68) and will not be reviewed here in detail. Early experiments with hypophysectomized animals indicated that little or no mammary development occurred unless anterior pituitary extracts were supplied. The anterior pituitary extracts employed prevented the atrophy of the gonads that accompanies hypophysectomy. Hence, it was suggested that the anterior pituitary hormones might act indirectly by restoring estrogen and progesterone secretion. A number of experiments, however, indicated that ovarian steroids administered alone were ineffective in stimulating mammary growth in hypophysectomized animals. These were interpreted as indicating that hormone(s) of the anterior pituitary acted directly in the regulation of normal mammary development. Numerous subsequent experiments clearly indicate that hormones of the anterior pituitary act directly upon the mammary gland. Lyons (40, 41) and, later, Mizuno and Hailo (48) reported

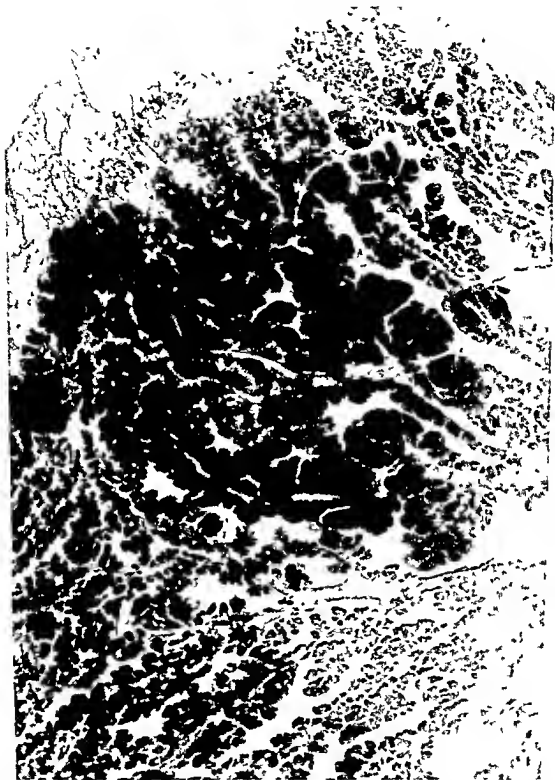


FIG 4 Two sectors of a mammary gland from a 5-months old oophorectomized rabbit pretreated with 20  $\mu$ g estrone plus 1 mg progesterone 5 days weekly for 4 weeks, and then after a 3 day interval with 0.1 mg (3 IU) of ovine prolactin in 1 ml of 2% butanol into the right sector, and only 1 ml of butanol into the left. Milk in the right sector could be seen through the skin, and could be expressed by 48 hours. No other sector of any gland secreted milk.  $\times 6$  By permission\* of W. R. Lyons *et al* (41) and Butterworths and Co., London.

that intraduct injections of prolactin into rabbits caused, in addition to secretory activity, considerable localized growth of the alveolar epithelium as assessed histologically and by DNA measurement (Fig. 4).

### C. INITIATION OF LACTATION

Three events which occur during the immediate, pre- and postpartum periods are essential to lactogenesis or the initiation of lactation. Although these events are highly interrelated, are regulated by the same hormones, and often occur almost simultaneously, it is convenient to separate them for purposes of discussion and experimentation. The first of these events is the formation of functionally differentiated secretory cells. This event apparently requires cell division and is characterized by the presence of the mammary cells' capacity to develop a characteristic enzyme complement and to synthesize milk components (2, 66). The second event essential to lactogenesis is development of the capacity for milk synthesis within newly formed, functionally differentiated cells. A portion of this development appears to be inherent, requiring only the formation of secretory cells and a hormonal environment consistent with maintenance of these cells. Another portion of this development seems to be dependent upon specific hormonally regulated processes (2, 66). The third essential event involves regulation of the expression of capacity for milk synthesis which results from the occurrence of the first two events (2, 7). *In vivo* experiments with hypophysectomized, ovariectomized, and/or adrenalectomized animals and *in vitro* studies indicate that the primary hormones required for lactogenesis are prolactin, cortisol or corticosterone, and insulin (Figs. 5-8), (2, 13, 23, 33, 58, 66). Prolactin appears to fulfill functions essential to secretory cell proliferation and differentiation during late pregnancy (1, 2, 4, 6, 18, 25, 34, 40, 41, 48, 66, 68). Recent *in vitro* data discussed by Topper and Oka (66) indicate that prolactin is not the mitogenic agent leading to secretory cell proliferation and differentiation, but rather, sensitizes mammary cells to the mitogenic actions of insulin and serum factors. In postmitotic or differentiated mammary cells, prolactin fulfills a central role in the regulation of RNA and protein synthesis and thus is essential for the development within newly formed cells of the (enzymatic) capacity for milk component synthesis (4, 6, 34, 66). Glucocorticoids regulate, in part, rates of synthesis of several enzymes essential to milk biosynthesis and are essential, along with insulin, for development of the extensive rough endoplasmic reticulum which is characteristic of fully developed mammary cells (7, 66). Mammary secretory cells are uniquely dependent upon insulin for their formation, development, survival, and function. As was noted above, prolactin appears to sensitize mammary cells to insulin



FIG. 5 Cortisol + insulin + prolactin 5 + 5 + 5  $\mu\text{g/ml}$ , respectively. All three hormones must be present in the medium to obtain this active secretory response. Note abundant accumulation of proteinaceous material in lumen of alveoli and fat secretion as indicated by lipid vacuoles in many cells and in the lumina  $\times 300$  kindly supplied by Dr. Joel J. Elias, Department of Anatomy, University of California, Berkeley, California.



FIG. 6. Cortisol + prolactin, 5 + 5  $\mu\text{g}/\text{ml}$ , respectively. In all media lacking insulin the tissue degenerates rapidly.  $\times 400$ . Kindly supplied by Dr. Joel J. Elias, Department of Anatomy, University of California, Berkeley, California.

during pregnancy leading to an insulin-induced wave of cell proliferation and, likely, differentiation—the first event essential to lactogenesis (2, 18, 66). During the development of secretory cells thus formed, insulin appears to fulfill several specific functions in synergy with prolactin and glucocorticoids (66). Differentiated mammary secretory cells are very dependent upon insulin for survival *in vitro* and *in vivo* (42, 66). When





FIG 7 Cortisol + insulin, 5 + 5  $\mu\text{g/ml}$  respectively. When these two hormones are present together, the tissue remains viable and shows some nonstainable clear secretion in the alveoli. Compare to Fig 5, in which the addition of prolactin induces the active protein and fat secretion response  $\times 300$ . Kindly supplied by Dr. Joel J Fliss, Department of Anatomy, University of California, Berkeley, California

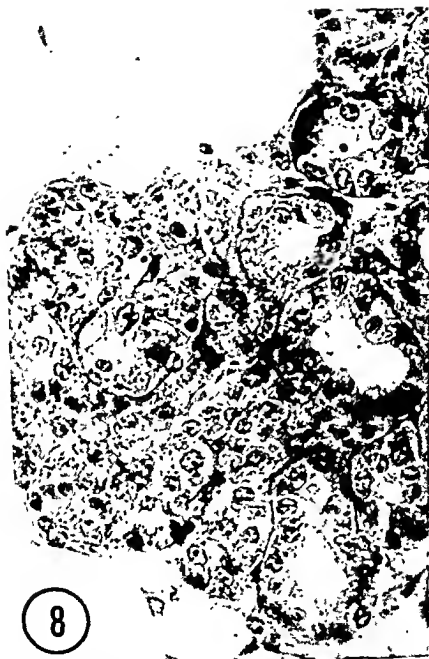


FIG. 8. Insulin + prolactin, 5 + 5  $\mu$ g/ml, respectively. This response is also produced by insulin alone. There is survival of many individual cells, but considerable loss of alveolar organization. Compare to Fig. 5, in which the addition of cortisol produces the active protein and fat secretion response.  $\times 400$ . Kindly supplied by Dr. Joel J. Elias, Department of Anatomy, University of California, Berkeley, California.

optimal milk secretion in hypophysectomized animals administered STH, prolactin, and cortisol. Some enhancement of production is obtained with addition of other hormones such as long-acting insulin, estrogens, and tri-iodothyronine. Treatment with these hormones does not completely restore milk yields, and it is not clear whether they exert their effects directly upon secretory tissue or indirectly by affecting general metabolism. After complete hypophysectomy, oxytocin must be administered to facilitate milk ejection. Otherwise, the mammary glands degenerate due to accumulation of milk in the glands. Prolactin and cortisol retard but do not prevent gland degeneration due to lack of oxytocin administration and milk removal.

Baldwin and Yang (7) and, later, Baldwin and Louis (5) summarized information relevant to specific actions of hormones upon mammary secretory cells during lactation. As was mentioned above, lactating secretory cells degenerate quickly *in vitro*. Thus, most data available on hormone actions during lactation result from *in vivo* studies with normal, endocrinectomized or anti-insulin injected animals and short-term *in vitro* studies with tissues removed from such animals. Also mentioned above was the fact that mature mammary secretory cells are extremely dependent upon insulin for normal function and survival. During acute insulin insufficiency *in vivo*, milk synthesis is reduced to very low levels within hours and cell degeneration becomes prominent within 1 or 2 days. The specific metabolic defects leading to cessation of milk synthesis within hours of insulin insufficiency are not known. The most limiting site of insulin action in mammary cells is not membrane transport or hexokinase function since cell concentrations of amino acids, glucose, and glucose 6-phosphate are elevated during acute insulin insufficiency. Also during acute insufficiency, cellular energy charge and redox state are altered. Insulin effects upon lipogenesis *in vitro* are dependent upon the redox state of the cell. These evidences suggest that insulin acts upon functions related to electron transport and energy metabolism. The exact sites of insulin action are unknown. Adrenalectomy of mid-lactating rodents results in a 40–50% decrease in milk production and decreases of 40 to 80% in activities of some twelve to fifteen enzymes closely associated with milk synthesis. Activities of many other enzymes are unchanged. These effects are reversed by glucocorticoid therapy. The depressions in enzyme levels are due, largely, to decreased rates of synthesis indicating that glucocorticoids regulate, in part, rates of synthesis of key enzymes involved in milk synthesis. Apparently, the depression in milk synthesis which occurs after adrenalectomy is not due to the reduced enzyme activities, since mammary metabolite data indicate that none of these become rate limiting. Indirect data indicate that flux through the phosphofructokinase reaction is reduced after adrenalectomy even though the

amount of phosphofructokinase in the gland is not reduced. Limited data indicate that prolactin acts in a general fashion upon lactating cells and is essential to maintenance of normal rates of RNA and protein synthesis. Much additional research will be required to establish the specific actions of hormones on mature secretory cells.

#### IV. Milk Synthesis

Considerable data are available regarding milk precursor:product relationships and metabolic pathways associated with energy metabolism and the synthesis of the major milk components. The data to be considered in subsequent sections were largely obtained through *in vivo* and *in vitro* experiments with isotope tracers, arteriovenous and blood flow measurements, and enzymatic techniques. The isotope tracer techniques have enabled investigators to determine the proportions and amounts of milk components formed from specific blood metabolites and to estimate the activities of specific metabolic pathways in mammary tissue. Studies based on blood flow measurements and determinations of metabolite levels in arterial blood entering, and venous blood leaving, the mammary glands provide a basis for quantitative estimation of the amounts of blood metabolites absorbed by the mammary gland and, if coupled with isotope techniques, assessment of the amounts of milk components formed from each blood metabolite. This approach requires accurate assessment of mammary blood flow rates and the venous blood obtained must be representative of the blood leaving the whole gland (38). Almost all mammary blood flow and arteriovenous (A-V) measurements have been made on ruminants because of the size and structure of their mammary glands. Enzymatic techniques have been employed primarily to characterize the enzymes and pathways of energy metabolism and of biosynthesis of milk components. Much useful data have been obtained on ruminant and nonruminant mammary metabolism using these techniques. Several recent and comprehensive reviews pertaining to various aspects of milk synthesis are available (21, 36, 55).

Precursor:product relationships for the lactating goat udder are summarized in Table I. The amount and composition of milk made is determined by the precursors taken up by the gland and the biochemical transformations which the precursors undergo in the mammary secretory cells. Figures 9 and 10 represent flow diagrams of the metabolic pathways involved in milk synthesis for rat and cow mammary glands. These two species differ greatly in precursors used for synthesizing milk components and the composition of milk produced. For example, rats make milk fat from glucose, while cows use acetate as the precursor of fatty acids syn-

TABLE 1

Major Precursor: Product Relationships for the Lactating Goat Udder

Precursor	A-V difference (mg/100 ml blood) <sup>a</sup>	Uptake (mg/ml milk produced) <sup>b</sup>	Product (mg/ml milk produced)
Glucose	14.4	69.6	Lactose 46
			CO <sub>2</sub> 17.4
			Glycerol 2.7
Acetate	5.7	27.5	Fatty acids 14.5
			CO <sub>2</sub> 12.9
Triglycerides	6.7	32.4	Triglyceride 29.0
Amino acids	1.04	5.0	Casein 4.6

<sup>a</sup> Arteriovenous (A-V) differences were obtained by subtracting mammary venous precursor concentrations from arterial concentrations.

<sup>b</sup> Uptake of precursors was calculated using a ratio of mammary blood flow to milk yield of 438:1 (31).

thesized in the mammary gland. Since details regarding metabolic pathways of milk synthesis are available in many biochemistry texts and cited references (21, 36, 55), the present discussion of milk synthesis will summarize data relating only to regulation of metabolic pathways, quantitative precursor:product relationships, and some aspects of energetic efficiency.

### A. LACTOSE SYNTHESIS

The primary blood precursor of lactose is glucose (38). Figures 9 and 10 summarize the pathway of lactose synthesis. There are two potential sites for regulation of lactose synthesis: glucose uptake and lactose synthetase. Evidence that glucose uptake is important include observations that low blood glucose concentrations result in low milk production (38). Lactose synthetase catalyzes the last steps in the pathway for lactose synthesis. The lactose synthetase complex consists of two proteins—galactosyltransferase and  $\alpha$ -lactalbumin. Galactosyltransferase is present in all tissues; however,  $\alpha$ -lactalbumin is found only in mammary tissue and is required for lactose synthesis. Current hypotheses regarding the regulation of lactose synthesis indicate that  $\alpha$ -lactalbumin is synthesized in response to hormonal stimuli. When this occurs,  $\alpha$ -lactalbumin combines with the galactosyltransferase in the Golgi and lactose is synthesized. Both lactose and  $\alpha$ -lactalbumin are then secreted (see Section II,A). Thus, lactose synthesis appears to be dependent upon rates of  $\alpha$ -lactalbumin synthesis (20).

Lactose secretion is involved in the regulation of water secretion in milk. Lactose in the alveolar lumen produces osmotic pressure which draws water and sodium from blood to milk. This suggestion is supported by the ob-

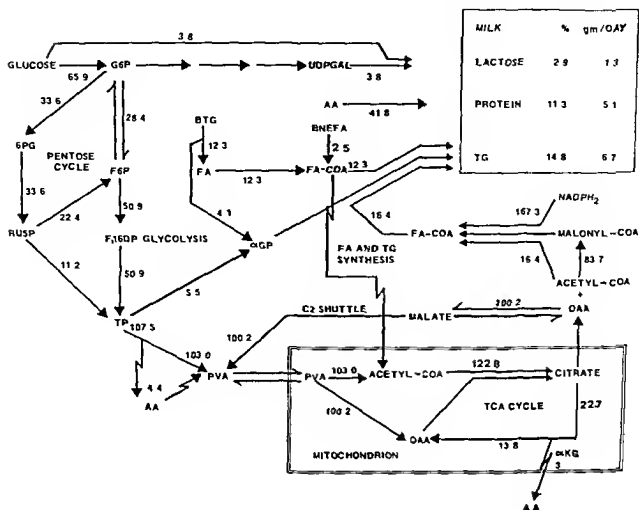


Fig 9 Metabolite fluxes during milk synthesis in the rat. Metabolite fluxes through the several pathways were computed on the basis of carbon, NADH, NADPH, and ATP requirements for synthesis of 45 gm milk/day, with the composition indicated. Fluxes for each reaction and/or pathways are expressed in mmoles per day. Rounding of errors in notating fluxes on arrows leads to slight imbalances but basically the system is in balance. Energy requirements were considered for maintenance but not for uptake, synthesis and/or secretion of minor milk components. The primary precursors of milk were considered to be blood glucose, triglycerides (BTG), free fatty acids (BNEFA) and amino acids (AA). Abbreviations include glucose-6-P (G6P), UDP galactose (UDPGAL), 6-phosphogluconate (6PG), ribulose-5-P (RU5P), fructose-6-P (F6P), fructose-1, 6 diphosphate (F1,6DP), triose phosphates (TP),  $\alpha$ -glycerol-P ( $\alpha$ GP), pyruvate (PVA), oxalacetic acid (OAA), fatty acid (FA), triglycerides (TG), tricarboxylic acid pathway (TCA), and fatty acyl-CoA (FACoA) (from Plucinski, 53).

servation that isolated goat mammary glands perfused without glucose secrete only small volumes of very concentrated milk (38).

## B PROTEIN SYNTHESIS

Casain and  $\beta$ -lactoglobulin make up about 90% of the protein of milk and are not found in blood; therefore, most milk protein must be synthe-

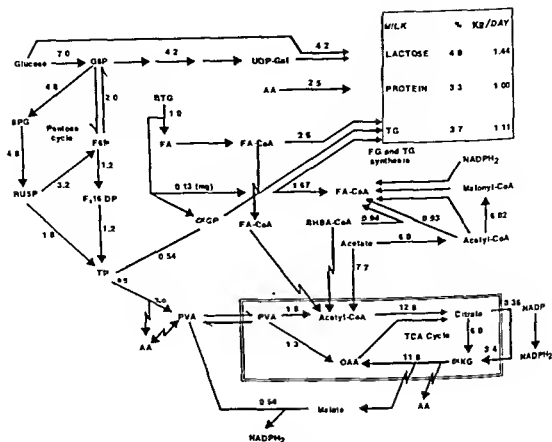


FIG. 10. Metabolite fluxes during milk synthesis in the cow. Fluxes were computed as described for rat in Fig. 9, with the exception that the primary precursors considered were glucose, acetate, amino acids, ketone bodies (BHBA), and blood triglycerides and fluxes are expressed as moles/day. Milk yield was considered to be 30 kg/day. Some metabolism of amino acids was considered and the secretion of 0.36 moles/day of citrate in milk was accommodated (from Baldwin and Yang, 7).

sized in the gland from amino acids (37). Mechanisms involved in milk protein synthesis are similar to those in other mammalian cells. Several reviews of protein synthesis mechanisms are available (24, 37).

Amino acid uptake and protein content of milk are affected by the nutritional status of animals and blood amino acid levels. Rook and Line (56), for example, found that heavy grain feeding to cows increased plasma glucose and amino acid levels and milk concentrations of casein and albumin. It is not clear, however, whether the increase in milk protein should be attributed to a greater availability of energy in the form of glucose or the greater availability of amino acids.

Recent reviews (24, 38) provide summaries of many references on the quantitative contributions of plasma amino acids to the synthesis of milk proteins in several species. These studies, based on A-V difference and

isotope tracer techniques, demonstrate that mammary uptake of amino acids from blood is sufficient to provide for milk protein synthesis in goats, cows, and sows. Furthermore, these amino acids are incorporated into milk protein. Some amino acids are taken up in excess of amounts needed for milk protein synthesis—arginine in the goat and cow and serine in the pig. These appear to provide nitrogen for the synthesis of several nonessential amino acids which are present in milk protein in amounts exceeding uptake.

### C. MILK FAT SYNTHESIS

A great many problems have been encountered in the investigation of milk fat biosynthesis, due largely to the complexity of and variation in composition of the milk triglycerides, the large number of potential and real precursors of milk fat found in blood, and the multiplicity of pathways for the synthesis and alteration of fatty acids in the mammary gland. The literature pertaining to these problems is much too extensive for specific consideration within the present context, and the reader desiring details is urged to consult recent reviews (7, 9, 15, 38).

The primary blood precursors of the fatty acids in milk fat triglycerides in ruminants appear to be acetate,  $\beta$ -hydroxybutyrate, and triglycerides of the low-density blood lipoproteins and chylomicra. Glucose in blood is not a significant source of carbon for milk fat or fatty acid synthesis. The primary blood precursors of milk fatty acids in nonruminants are glucose and blood triglycerides. Table 11 presents a summary of numerous data on the contributions of the primary precursors of the fatty acids in cow milk triglycerides. Only the major fatty acid components of milk fat are represented.  $\beta$ -Hydroxybutyrate (BHBA) is a major blood precursor of butyrate and the first four carbons of  $C_6$  to  $C_{10}$  fatty acids.

Figures 9 and 10 summarize the pathways of fatty acid synthesis in rat and cow mammary glands. Represented are synthesis via the malonyl-CoA pathway in rats and synthesis via the malonyl-CoA pathway and chain elongation (BHBA + acetyl-CoA) pathway in cows. More detail on the specific pathways and mechanisms of NADPH<sub>2</sub> synthesis in rats and cows (Figs. 9 and 10) can be found in a recent review by Bauman (9). An intriguing feature of mammary fatty acid synthesis is the formation of a mixture of fatty acids varying in chain length from  $C_{10}$  to  $C_{16}$ . In other tissues the primary product is palmitate ( $C_{16}$ ). Purified mammary fatty acid synthetase forms palmitate. The mechanism causing shorter chain fatty acid synthesis *in vivo* and in tissue homogenates is not clear and is currently receiving considerable attention. Blood triglycerides in chylomicra and low-density lipoproteins are hydrolyzed by lipoprotein lipase in the



TABLE II  
Precursors of Milk Fat Fatty Acids\*

Fatty acid	Mole % in milk fat	Grams/100 gm milk fatty acid from	
		Acetate + BHBA	Blood triglyceride
C <sub>4</sub>	10	4.0	—
C <sub>6</sub>	4	2.1	—
C <sub>8</sub>	2	1.3	—
C <sub>10</sub>	5	4.0	—
C <sub>12</sub>	3	2.8	—
C <sub>14</sub>	10	5.2	5.2
C <sub>16</sub>	25	14.1	14.1
C <sub>16:1</sub>	3	—	3.6
C <sub>18</sub>	8	—	10.5
C <sub>18:1</sub>	27	—	35.0
C <sub>18:2</sub>	3	—	3.5

\* Adapted from Smith (62).

capillaries prior to uptake by the gland. Observations indicating this mechanism have been obtained in A-V difference studies using lactating goats and in histochemical studies with lactating mice (38). Triglyceride glycerol in milk fat is formed from blood triglyceride glycerol and blood glucose. Several data indicate that glycerol from blood triglycerides is quantitatively converted into glyceride glycerol. Additional required glycerol is synthesized from glucose in both ruminants and nonruminants (9, 38).

Milk fat is the most variable component of milk, and because of this, and the fact that depressions in percentage milk fat in the milk of cows fed high concentrate and other diets are a problem of considerable practical importance, factors affecting milk fat synthesis have been studied extensively. It appears that milk fat synthesis is affected by the general metabolic status of the animal and by intracellular regulatory mechanisms in the mammary gland (16).

#### D. EFFICIENCY OF MILK SYNTHESIS

The data presented in Tables III and IV were prepared to enable estimation of efficiencies of milk synthesis in the mammary gland. The calculations are based on mammary uptake data, milk composition data, and consideration of mammary metabolic pathways as presented in Figs. 9 and 10. Uptakes for Table III were calculated using, primarily, input requirements and metabolic pathways because rat A-V difference data are limited.

TABLE III

Estimation of the Efficiency of Milk Synthesis in the Rat Mammary Gland<sup>a, b</sup>

	Uptake/100 gm milk			Output/100 gm milk	
	mmoles	kcal		mmoles	kcal
Glucose	155.0	104.3	Lactose	8.4	11.3
Amino acids	102.7	58.2	Fat	21.3	143.7
Triglyceride	11.0	83.5	Protein	102.7	58.5
		246.0			213.5

<sup>a</sup> Efficiency = output/uptake  $\times$  100 = 213.5/239.9  $\times$  100 = 87%.<sup>b</sup> Adapted from Plucinski (53)

TABLE IV

Estimation of the Efficiency of Milk Synthesis in the Cow Mammary Gland<sup>a, b</sup>

	Uptake/kg milk			Output/kg milk	
	mmoles	kcal		mmoles	kcal
Glucose	373	251	Fat	48	304
Acetate	525	110	Lactose	140	189
BHBA	52	27	Protein	250	143
Triglyceride	30	213	—	—	—
Amino acids	250	142	—	—	—
		743			636

<sup>a</sup> Efficiency = output/uptake  $\times$  100 = 636/743  $\times$  100 = 86%<sup>b</sup> Adapted from Baldwin and Yang (7)

Extensive A-V difference data were used in formulating Table IV. The estimates obtained indicate that milk synthesis is a very efficient process. Efficiencies of milk synthesis for whole animals are less than those for the gland alone because energy is lost during substrate transformation in extra-mammary tissues. However, whole animal data support the view that mammary milk synthesis occurs at close to theoretical (biochemical) efficiencies (12).

## V. Milk Ejection

Milk secreted by the alveolar cells of the mammary gland cannot be removed until the myoepithelial cells surrounding the alveoli contract and

force the milk from the alveoli and small ducts to the large ducts and the gland and teat cisterns. The neuroendocrine reflex activated by suckling or milking, which regulates myoepithelial cell contraction and milk ejection, has been the subject of extensive investigation. The development of knowledge concerning milk ejection was treated in a very interesting review by Folley (23). Recent advances regarding the functional innervation of the mammary glands, the hypothalamo-neurohypophyseal system, and the role of oxytocin in milk expulsion have been reviewed in detail (17, 26).

#### A. FUNCTIONAL INNERVATION OF THE MAMMARY GLANDS

The innervation of the mammary glands resembles that of the skin, as might be expected in view of their common embryological origin, and is comprised of somatic sensory and sympathetic motor fibers arising from different segments of the spinal cord, depending upon whether a given species has thoracic, abdominal, and/or inguinal mammary glands. There has been some controversy regarding the presence or absence of parasympathetic innervation in the udders of ruminants, but, at present, no convincing evidence of such innervation is available (14). The sensory nerves are distributed, primarily, in the skin surrounding the mammary glands and are present in exceptionally large numbers in the teats. The supply of sensory nerves to the mammary parenchyma appears to be very limited. The motor nerve endings found in the mammary glands supply, primarily, the blood vessels, the connective stroma, the large ducts, and the muscles surrounding the teat and gland cisterns and the teat sphincter (14). Stimulation of the peripheral extremities of the mammary nerves and administration of adrenaline cause vasoconstriction, rhythmic contractions in the teats, and relaxation of smooth muscles surrounding the teat and gland cisterns. These observations and others mentioned later support the contentions that the autonomic fibers supplying the mammary gland are sympathetic and adrenergic and that motor endings do not supply the myoepithelial cells surrounding the alveoli and small ducts. Hence, motor elements are not responsible for milk ejection.

#### B. REGULATION OF OXYTOCIN RELEASE

The sensory stimuli associated with suckling or milking lead to the liberation of the neurohormone, oxytocin, and possibly vasopressin into the blood. Considerable attention has been focused on examination of the efficiency of various visual, conditioned, and physical stimuli in evoking oxytocin release and subsequent milk ejection in the course of formulation of recommendations concerning proper, practical premilking practices for

dairy cattle and goats. These studies have resulted in the realization that practices which cause vasoconstriction (as by adrenaline) in the udder prevent milk ejection, due to the prevention of oxytocin entry to the udder and that the most effective stimulus to milk ejection is manipulation of the teats during suckling or the act of milking. In this connection, it is surprising that the types of stimuli (e.g., tactile, thermal) which excite the sensory nerve endings in the teats have not been characterized in detail. However, there is general agreement that excitations resulting from the complex of stimuli associated with milking and suckling are transmitted to the neurohypophysis and cause oxytocin release. Transmission of excitations from the teats involves a complex of ascending spinal paths and, possibly, medullary paths which analyze, integrate, and modulate these afferent impulses to the supraoptic and paraventricular nuclei of the hypothalamus, which in turn, transfer the signal to the neurohypophysis (17, 26). The supraoptic and paraventricular nuclei perform two functions related to the release of oxytocin from the neurohypophysis. Oxytocin and vasopressin (ADH) are synthesized in these hypothalamic nuclei and are transported to the neurohypophysis. Oxytocin and vasopressin thus synthesized and transported are stored in the neurohypophysis and released in response to nervous stimulation of and from the supraoptic and paraventricular nuclei. It should be pointed out that stimulations of the teats are not the only cause of oxytocin release. Other common stimuli include those arising from the genitals, a basis for some historical milking practices described by Cowie and Tindal (13) and Folley (23), and from the injection of hypertonic saline. This latter observation emphasizes the fact that stimuli causing oxytocin and vasopressin release from the posterior pituitary have a common origin in the hypothalamus of many species. The role of the hypothalamus in the regulation of the secretion of adenohypophyseal hormones involved in lactation is described in Chapter 3.

### C. MILK EJECTION

Oxytocin released by the neurohypophysis is transported by the blood to the mammary glands, where it acts upon the myoepithelial cells surrounding the alveoli and ducts of the glands and causes them to contract and expel the milk. Oxytocin also causes relaxation of the smooth muscles surrounding the large ducts and gland and teat cisterns, thus providing for enlargement of these structures to accommodate the milk ejected from the alveoli. Intramammary pressure rises as a result of the forcible ejection of milk from the alveoli and small ducts, and the suckling young of the milker has only to overcome the resistance of the teat sphincter in order to accomplish the final stage of milk removal (23). In many animals the buildup of

intramammary pressure is sufficiently great to overcome the resistance of the teat sphincter and forces milk to drip or spurt from the teats (23).

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# 15 Reproduction in Horses

G. H. Stabenfeldt and J. P. Hughes

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## I. Introduction

The great increase in the understanding of reproductive physiology of the mare and stallion which has occurred during the last 10 years is due

to the development of new methodological techniques, particularly in the area of reproductive endocrinology, coupled with increased attention to clinical findings. The First International Symposium Upon Equine Reproduction (80) held in Cambridge, England during July 1974, contributed significantly to the advancement of knowledge of horse reproduction and it is a must for anyone seriously interested in this subject. The reader is also referred to Andrews and McKenzie (7), Berliner (14), and Nishikawa (68) for a review of the earlier work in horse reproduction.

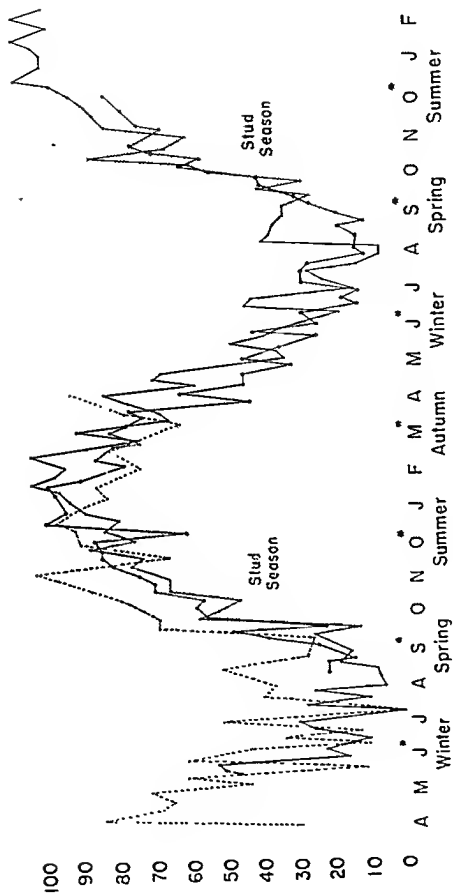
A major problem in horse reproduction is that the physiological reproductive season does not necessarily coincide with the breeding season imposed on equidae by many breed associations, wherein all foals become 1 year of age on January 1. In order for horses to compete at the track or in the horse show arena as yearlings or as 2 and 3 year olds, the breeding season must commence in February or early March at a time when normal ovarian activity may not have been reestablished.

Another problem associated with horse reproduction is that little selection for reproductive performance has been practiced in either the mare or the stallion. The predominant value of the horse has been determined through competitive excellence. Time may bring changes in the rules of horse breed associations on the dating age of foals, which, hopefully will align the imposed and physiological breeding seasons, but it is likely that speed and performance will continue to determine which animal the owners will use in their breeding programs.

## II. Estrous Cycle

Most mares are seasonally polyestrous, undergoing periods of ovarian inactivity most often associated with the onset of the winter season. These periods of ovarian inactivity can last from 2 to 6 months, depending on the latitude and possibly the climate of the particular locale. The further north or south of the equatorial line, the more pronounced the occurrence of winter anestrus. Some mares cycle the year around. Figure 1 presents data which suggest that 20-25% of the mares in Australia cycle during the period of winter anestrus (71). In one 2-year study of eleven mares at Davis, California, five mares underwent winter anestrus, five mares cycled continuously during the 2 years, and one mare cycled continuously the first year and underwent anestrus the next year (55).

Light is an important factor which influences the sexual season of the mare (Fig. 1). Although the mare responds to decreasing light by terminating cyclical ovarian activity, the response is slow and may lag 4-6 months after the summer solstice. Similarly, her response to increasing



o----o 1959-60 Avg 7.5 mares/wk  
 ●—● 1961-63 — 24 — —  
 ○—○ 1964-65 — 37 — —

FIG. 1.  $\Delta$  frequency distribution curve for the seasonal occurrence (percentage) of ovulation in mares obtained from three surveys in Australia. Note the low ovulation rate associated with the start of the breeding season. The asterisk (\*) refers to the summer and winter solstice and the vernal and autumn equinox. (From Osborne, 71.)

light, and cyclical ovarian activity in the spring is not reestablished until 3 to 5 months after the winter solstice. Increasing the photoperiod with artificial lighting during the winter is utilized to bring about the resumption of ovarian activity earlier in the spring (60).

## A. LENGTH

There is considerable variation in the literature concerning the length of the estrous cycle of the mare (7, 32). Prolonged estrus without ovulation and erratic estrous behavior in the early spring account for much of this variation (54, 55). In addition, spontaneous prolongation of luteal activity occurs rather commonly in the nonpregnant mare (4, 54). Ovulatory intervals determined by rectal palpation, together with psychic signs of estrus when teased by a stallion during the physiological breeding season, indicate that the average length of the estrous cycle is 21–22 days (55).

## B. OVARIAN STRUCTURE AND FUNCTION

### 1. Folliculogenesis

While definitive follicle growth and ovulation of one follicle occurs during estrus, it is not uncommon for other follicles to continue to grow with the following possible fates: (1) ovulation within 24 hours of the first ovulation, (2) ovulation during the luteal phase of the cycle, (3) eventual regression during the luteal phase (55). Data have been presented suggesting that waves of follicular growth and estradiol synthesis occur at 10-day intervals, irrespective of a functional corpus luteum being present in the mare (37, 100).

The magnitude of multiple follicle development in the mare is illustrated by the finding of two or more follicles in 125 of 276 estrous periods that were greater than 25 mm in diameter (55). Only occasionally did the smaller follicle at the onset of estrus become either the follicle ovulated or the first of several ovulations (6%) (66).

Bengtsson and Knudsen (11) and van Niekerk and van Heerden (99) have emphasized the importance of adequate nutrition for the reestablishment of follicular growth in the spring.

The main secretory product of the follicle, as in other domestic species, is estrogen. Estrogen levels (Fig. 2) increase in the peripheral plasma prior to and during sexual receptivity (67, 70, 72).

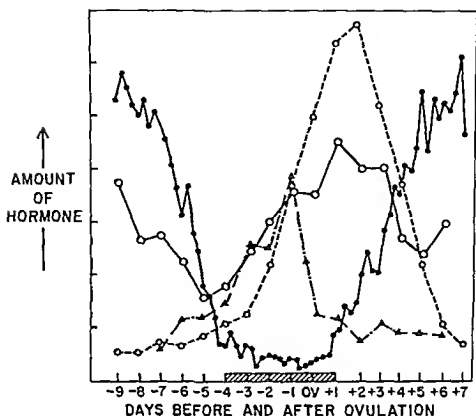


FIG. 2. The endocrine changes found in the peripheral plasma of mares in association with ovulation (OV). The progesterone values (●—●) are from Stabenfeldt *et al.* (87) (one vertical division = 1 ng/ml plasma); estradiol values (Δ—Δ) are from Noden *et al.* (69) (one vertical division = 5 pg/ml plasma); LH values (○---○) are from Geschwind *et al.* (41) (one vertical division = 2 ng/ml plasma); and the FSH values (⊙—⊙) are from Evans and Irvine (37) (one vertical division = 20 ng/ml plasma). The horizontal bar represents the period of sexual receptivity (estrus).

## 2. Ovulation

As in other mammalian species, the embryonic equine ovary begins its development with cortical tissue surrounding the medullary portion in a normal arrangement. As the equine ovary develops in the neonatal period, the cortical tissue (germinal epithelium) becomes confined to one area and is nearly surrounded by medullary tissue at the time the foal is 4 months of age (65, 87). As a result of this arrangement, mares ovulate follicles from one specific area of the ovary, i.e., the ovulation fossa (Fig. 3). The tunica albuginea, which separates the cortical and medullary areas in the embryonic gonad, eventually covers most of the external surface of the adult equine ovary but is absent from the germinal epithelial layer through which ovulation occurs (65).

Follicles usually reach a size of 35 to 55 mm prior to ovulation. While perceptible softening of the follicle has been reported during the last 24

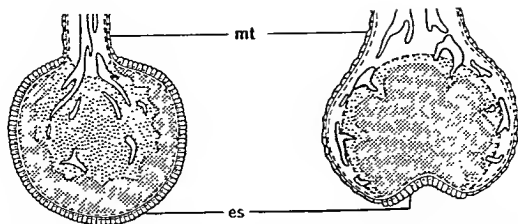


FIG. 3. Diagrammatic comparison of the corticomedullary arrangement in the mature equine ovary (right) and other mammalian ovaries (left): es, epithelium superficiale; mt, mesothelium; close stipple, cortex; open stipple, medulla. Note the reversal of corticomedullary areas with only a small area of contact of the cortical area with the ovarian surface (ovulation fossa). (From Mossman and Dukes, 65.)

hours prior to ovulation, it was noted only 28% of the time in one study in which ovarian palpation was performed at 24-hour intervals (55).

The time of ovulation is closely related to the cessation of estrus as the majority of mares are out of heat within 48 hours after ovulation (Fig. 4). In one study involving 353 ovulations, 45.9% occurred the day before the end of estrus while 32.0% occurred 48 hours prior to the end (55). During the normal breeding season, the cessation of sexual receptivity after an estrus of normal length, usually indicates that ovulation has occurred. Ovulatory failure during the breeding season (physiological) appears to be rare in that only one case of ovulatory failure could be documented in one series of approximately 300 estrous periods (unpublished).

A high incidence of multiple ovulation has been observed in mares including reports of 25% (86) and 14.5% (71), although 3.8% was reported in one study (7). Most of the multiple ovulations occur over a fairly short time interval. In one study of 83 multiple ovulations which were recorded during estrus, 62 (75%) occurred within 24 hours and 75 (90%) occurred within an interval of 48 hours (55). The occurrence of multiple ovulation is rare in pony mares (42).

Follicles are occasionally ovulated during the luteal phase of the cycle (54, 86). While the incidence of luteal phase ovulation is not precisely known, it probably occurs in about 5% of the estrous cycles. The interval from the initial ovulation to the luteal phase ovulation may be as short as 2 and as long as 12 days (36, 41). The follicle development which leads to ovulation during the luteal phase of the cycle is not accompanied by cervical changes or manifestations of estrus. The fertility of luteal phase ovulations is not known.

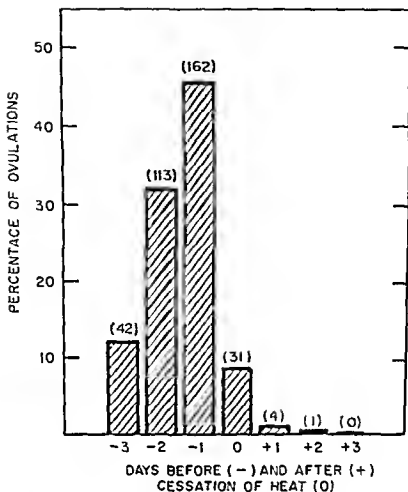


FIG. 4. The relationship of the time of ovulation and the end of the sexual receptivity. The numbers (in parentheses) on the vertical bars indicate the number of observations.

The gonadotropin patterns associated with the estrous cycle of the mare are considerably different as compared to other mammalian species. In most mammals studied to date a short-lived surge of gonadotropins occurs 12–24 hours prior to ovulation (see Chapter 2). The mare, in comparison, has a prolonged rise and decline of LH which occurs over a period of about 10 days (41, 69, 72, 104) (Fig. 2). The rise in LH begins several days before ovulation and continues for several days postovulation.

LH may be sensitive to progesterone feedback control in that it does not begin to rise until after the regression of the corpus luteum is complete (86). However, high circulating progesterone levels do not constitute an effective negative feedback control mechanism once LH release has begun. One explanation for the extended LH rise in the mare is that the release pattern of gonadotropins may be prolonged over several days versus a rapid release over a period of hours in other species. Another explanation for the prolonged LH rise is that equine LH has a longer half-life than reported for most species (44).

The pattern of FSH during the estrous cycle of the mare is shown in



Fig. 2; two peaks were noted during the cycle, one during late estrus/early diestrus and one at mid-diestrus (37). FSH and LH followed similar patterns during estrus and early diestrus. The increased FSH levels observed during late estrus/early diestrus coincide with follicular growth that often occurs immediately after estrus (55, 75, 100).

### 3. Luteal Function

Initial significant luteal activity, i.e., the production of progesterone, begins about 24 hours after ovulation (87, 100) (Fig. 2). Peak functional activity is reached about 6 days postovulation. There is good agreement that the corpus luteum life span of the mare lasts approximately 14 days (66, 100).

Termination of luteal function is abrupt with regression occurring over a period approximately 30 hours (H. Kindahl, personal communication). A significant rise in a metabolite of  $\text{PGF}_{2\alpha}$  noted in conjunction with luteal regression (Fig. 5) suggests that prostaglandins are important for the termination of luteal function in the nonpregnant mare.

Uterine control of cyclic luteal activity in the nonpregnant mare has been demonstrated by hysterectomy (43, 89). Removal of the uterus during the luteal phase of the cycle results in the persistence of luteal activity. The precise length of luteal function in the hysterectomized mare has been difficult to determine because luteal phase ovulations occur also in these

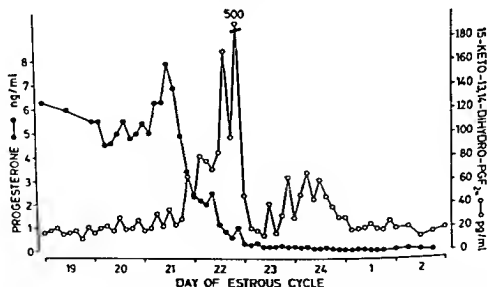


FIG. 5. The relationship of luteal regression in the mare as indicated by progesterone concentration (●—●) and the release of  $\text{PGF}_{2\alpha}$  (○—○) as indicated by changes in its main metabolite, 15-keto-13,14-dihydro- $\text{PGF}_{2\alpha}$ . Day 1 is the first day of sexual receptivity (57a).

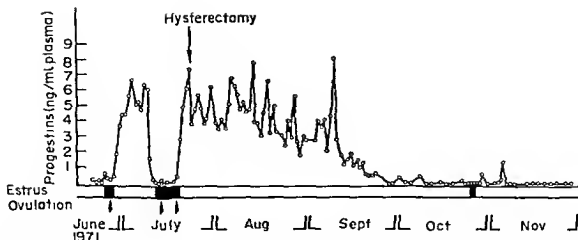


FIG. 6. Progestin levels in the plasma of a mare during the estrous cycle before hysterectomy and following hysterectomy on day 4 after ovulation during the next cycle. Horizontal bars indicate the period of sexual receptivity. Vertical arrows indicate the occurrence of ovulation. (From Stabenfeldt *et al.*, 89.)

mares. One mare that did not have additional ovulations had significant luteal activity for approximately 2 months following hysterectomy on the 4th day of the luteal phase of the cycle (Fig. 6).

The corpus luteum of the mare is palpable for an average of 8.9 days postovulation (55). Following ovulation, the follicular area usually fills with blood by 10–12 hours postovulation with redistension sometimes occurring to the follicles' preovulatory size. This structure is sometimes indistinguishable from the original follicle, although most postovulatory structures develop a spongy to rubbery consistency. The consistency later becomes firmer in texture (see Fig. 7 for a cross-sectional view of the corpus luteum).

## C. TUBULAR GENITALIA

### 1. Uterus

The uterus of the mare is somewhat T-shaped due to the fact that the ovaries are suspended in a lateral position in the upper part of the posterior abdominal cavity. Biometric changes of the reproductive system have been reported (102).

The response of the uterus to gonadal hormones differs in the mare and cow. The uterus of the mare has only slight tone and tubularity during estrus as contrasted to the distinct tubularity found in the cow. Within 2 or 3 days after ovulation, tone and tubularity increase, but not to the extent of the response of the bovine uterus during estrus, and is variably maintained in this state until regression of the corpus luteum begins. The well-defined increase in tone and tubularity at 21 days postbreeding is sometimes used as a presumptive diagnosis for pregnancy (97).

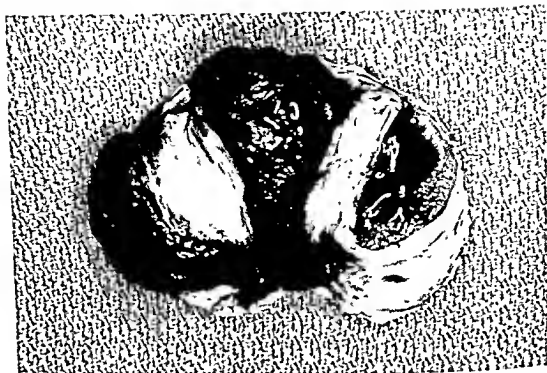


FIG. 7. A cross-sectional view of the ovary of a mare 2 days after a double ovulation. Note the wedge-shaped corpora lutea at the left extremity and middle of the ovary with the constricted portion directed toward the ovulation fossa. A large follicle in cross section is on the right extremity.

## 2. Cervix

The cervix of the mare is very responsive to estrogen secretion during the time of sexual receptivity. The cervix is relatively short and firm during diestrus with the folds of the external os being well-defined. At the beginning of estrus the cervix begins to relax slightly and the folds of the external os become edematous and less well-defined. The folds continue to enlarge and the cervix is soft and relaxed until the end of estrus. The external os becomes hard and budlike in appearance and the cervix becomes firm and narrow by the 17th day of gestation (83).

Visual examination of the external os of the cervix is often done in conjunction with rectal palpation of the cervix and ovaries for the purpose of predicting the time of ovulation (54).

## D. ESTRUS

### 1. Duration

The duration of estrus in the mare during the physiological breeding season has been reported as averaging 6 days with a range of 2 to 11 days

(68). Trum (96) reported 61% of approximately 1500 estrous periods were 4–6 days in length. This agrees with a more recent study involving 293 estrous periods in which the average period for sexual receptivity was 5.7 days (55).

Periods of sexual receptivity can be very irregular in length and timing at the beginning of the breeding season. Once ovulation has occurred, the duration of estrus becomes more regular. The estrus associated with the first ovulation of the season is often longer than subsequent estrous periods. An ovum ovulated after a prolonged period of estrus is just as viable as one ovulated after a 5- to 6-day estrus. The duration of estrus tends to become progressively shorter during the first 2 or 3 months of ovarian cyclicity (55).

The onset of sexual receptivity in the mare can occur over a period of several days with a gradual increase in intensity (7). This is in contrast to the cow, sow, and ewe in which full manifestations of estrus are achieved within a matter of hours. The termination of estrus is more abrupt, with the secretion of significant amounts of progesterone by the developing corpus luteum, 24 hours postovulation being the critical determining factor (Fig. 2).

## 2. Psychic Manifestations

While mares do not exhibit the homosexual tendencies seen in the cow during estrus, they often seek contact with other mares and geldings. Mares in estrus show certain typical behavioral attitudes when exposed to a stallion, or even other mares or geldings (8). Typically, the mare assumes a urinary stance in which the hind legs are flexed and the tail is raised. Small amounts of urine are often expelled and the clitoris is exposed in a rhythmic fashion. The staining of the vulvar lips and the posterior part of the hind legs with urine and its salts through the release of small amounts of urine at close intervals suggests that a mare is in estrus.

Mares will occasionally fail to manifest estrus (Fig. 8) in conjunction with normal follicle development and ovulation (7, 55). These mares should conceive if inseminated at the proper time.

## 3. Abnormal Sexual Behavior

Mares have been reported to show stallionlike behavior and actively tease other mares. These mares exhibit some of the features of the stallion, including the typical arching of the neck, vocal sounds, and eversion of the upper lip during the teasing process. The basic cause for this type of abnormal behavior is not known in all cases, but it has been observed in

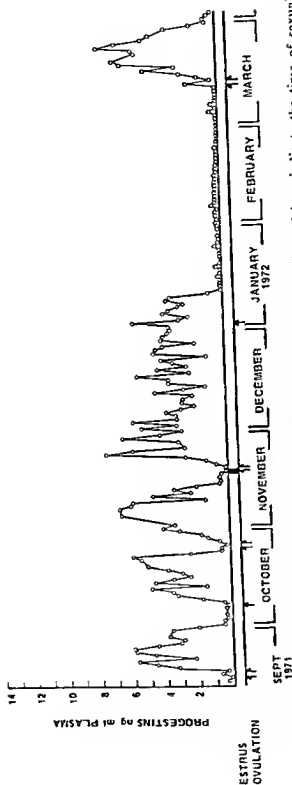


FIG. 8. Peripheral plasma progesterin levels in a nonpregnant mare. The black horizontal bars indicate the time of sexual receptivity. The arrows indicate the occurrence of ovulation. Note the absence of sexual receptivity (except for one day) in spite of regular ovarian activity, the occurrence of a prolonged corpus luteum (December), and the occurrence of anestrus (January to March). (From Stabenfeldt *et al.*, 88.)

conjunction with the occurrence of granulosa cell tumors. These tumors often produce androgenic hormones that may be responsible for the male-like behavior (unpublished) (91). Other mares with granulosa cell tumors may be anestrus.

## E. UTERO-OVARIAN RELATIONSHIPS

### 1. Intrauterine Infusion

It is now apparent that intrauterine saline infusion produces estrus through the premature regression of the corpus luteum. It has also been shown that the corpus luteum must be of a certain maturity (4 days of age) for luteolysis to be initiated (66) (Fig. 9). This degree of maturity which is required is apparently due to the developing corpus luteum being refractory to the effects of a luteolysin and is not the result of the uterus failing to release a luteolysin. Allen and Rowson (5) have shown the newly developing corpus luteum to be unresponsive to an analog of  $\text{PGF}_{2\alpha}$ , a

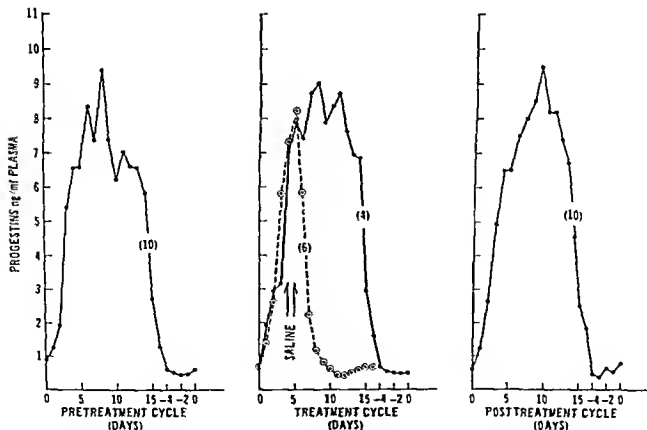


FIG. 9. The effect of intrauterine saline infusion on corpus luteum life span (as indicated by plasma progesterone levels) on the 4th or 5th day postovulation. The numbers in parentheses represent the numbers of animals. The numbers at the base of the graph represent an adjusted 21-day cycle length with day zero as the day of ovulation. (From Neely *et al.*, 66.)

known luteolytic drug. The mechanism involved in induction of luteal regression by saline infusion is unknown, but it is likely to be through the premature release of a luteolysin from the uterus. Progesterone analyses done in conjunction with saline infusion have shown that regression begins within 24 hours after infusion (66).

The intrauterine infusion of stallion semen during diestrus is followed by estrus within a few days, apparently due to the termination of luteal function (23). These authors suggested the presence of  $\text{PGF}_{2\alpha}$  in stallion semen may be important for this effect.

## 2. Spontaneous Prolongation of Luteal Function

Spontaneous prolongation of luteal function has been reported in mares that are free of uterine anomalies and infection (4, 54, 88) (Fig. 8). The average duration of persistence of the luteal phase is 2 months. Follicle development often continues during the persistent luteal phase without causing estrus. Ovulation is occasionally observed during these periods. Luteal function is finally terminated abruptly and cyclic ovarian activity is then resumed. This syndrome may be initiated by inadequate luteolysin release at 13 or 14 days postovulation. The reason for this deficit is not known, although it may be due to a failure of endometrial cells to synthesize and/or release adequate amounts of luteolysin at the proper time. Earlier reports of prolonged diestrus were probably caused by the prolongation of luteal activity (7, 34).

Persistence of the corpus luteum constitutes a very important reproductive problem in the breeding of mares as it decreases the number of exposures a mare can have to a stallion within the breeding season. The failure of a mare to return to estrus if not bred, following a nonfertile mating or a fertile mating with early embryonic loss, is a prime reason for suspecting the presence of a persistent corpus luteum. Blood levels of progesterone will confirm the diagnosis.

Mares with persistent corpora lutea often do not respond to the intrauterine infusion of saline (unpublished), which suggests that the syndrome is caused by uterine malfunction, i.e., a failure to release a luteolysin in response to saline infusion. The persistent corpus luteum is responsive to systemically administered prostaglandins (4).

## 3. Uterine Infection

An acute inflammatory response of the uterus during the luteal phase of the cycle can result in the shortening of the cycle through the initiation of premature regression of the corpus luteum (56). Whether this response is mediated through the release of uterine luteolysin is not known.

Chronic infections leading to endometrial destruction in the mare can result in the prolongation of the estrous cycle (16). Continual luteal function for 20 months has been observed in a mare with extensive destruction of the uterine endometrium (unpublished). In all cases observed by the authors, the prolongation of the estrous cycle was caused by a failure of the corpus luteum to regress at the normal time. The mechanism of action appears to be failure of adequate luteolysin release in that there appears to be a quantitative relationship between the amount of endometrial tissue present and the capability for luteolysin synthesis and release (unpublished).

### III. Pregnancy

#### A. RECOGNITION OF PREGNANCY

In many mammals the oviduct distinguishes fertilized from unfertilized ova. Several researchers have shown that only fertilized horse eggs reach the uterus with unfertilized ones retained in the oviducts (18, 90, 98).

#### B. ENDOMETRIAL CUPS

The reader is referred to Chapter 12 for a discussion of the formation of cups. Their formation is unique to the Equidae, and is unusual in that cells from the fetal placenta detach themselves, invade the endometrium, and establish isolated endocrine organs of temporary function (3, 29).

A number of factors have been found to govern the production of PMSG: size, parity, number of fetuses present, and genotype of the fetus. However, almost certainly other agents also influence PMSG levels in blood. Higher PMSG levels were noted in serum of ponies as compared to larger breeds of horses (28). While age does not influence PMSG production, parity is a factor. A reduction in PMSG concentration in successive pregnancies was noted in Shetland ponies (79) but not in thoroughbreds (1). Higher levels of PMSG were found in the serum of mares carrying twin fetuses if they were in opposite horns of the uterus and each with a set of endometrial cups (79). A mare carrying a mule fetus has a much lower concentration of the hormone than if she were carrying a horse fetus, convincing proof that fetal genotype influences PMSG production (27).

The endometrial cups have some autonomy of function concerning PMSG synthesis in that secretion continues even if the fetus is surgically removed (1), or dies (63). Thus it is possible to have a positive diagnosis of pregnancy in the absence of a viable fetus when immunological or biological



tests for pregnancy (PMSG) are used. If the corpus luteum is regressed with prostaglandins in nonpregnant mares with actively secreting endometrial cups (2, 4), the mare frequently fails to return to estrus in spite of high circulating levels of PMSG.

## C. MAINTENANCE OF PREGNANCY

### 1. Primary and Secondary Corpora Lutea

It has been shown by van Rensburg and van Niekerk (100) and Squires and Ginther (85), through endocrine analysis and structural studies, that the primary corpus luteum of pregnancy (corpus luteum formed following the ovulation involved with fertilization) does not regress around the 40th day of gestation, as often reported, but is active for at least the first 3 months of gestation, and regresses in conjunction with secondary corpora lutea during the 5th–6th month of gestation (31, 84) (Fig. 10).

Secondary corpora lutea are formed during pregnancy usually between the 40th and 60th day of gestation (31, 48). Secondary corpora lutea are

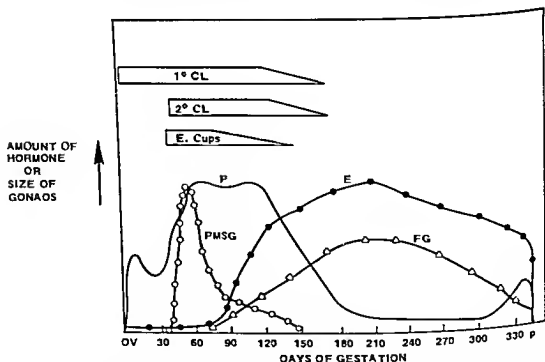


FIG. 10. The endocrinology of pregnancy in the mare. The progesterin (P) values (—) and PMSG values (O—O) are from Hollan *et al.* (48). The estrogen (E) values (●—●) are from Nett *et al.* (67). FG, fetal gonads ( $\Delta$ — $\Delta$ ); E. Cups, endometrial cups; 1° CL, primary corpus luteum; 2° CL, secondary corpora lutea.

apparently formed by either ovulation (32%) or luteinization of follicles (68%) (84).

## 2. Placenta

Placental production of progesterone probably begins in a gradual manner during the first trimester of pregnancy and becomes the primary source of progesterone production by the time all corpora lutea have regressed (85). Ovariectomy at 200 days of gestation has shown that the ovaries were not essential for pregnancy after this time (47). Although progesterone levels decline in the maternal peripheral plasma by midpregnancy, levels remain high within the feto-placental unit (81). A slight rise in peripheral levels of progesterone occurs during the last 30 days prepartum (48) (Fig. 10).

There is a definite rise and fall in estrogens at 37 to 38 days of gestation (E. Palmer, personal communication). Estrogen secretion later in pregnancy is probably a function of the placenta because estrogen levels rise at 90 days of gestation after ovarian follicular activity has subsided (67). In addition, ovariectomy at day 200 of gestation did not affect the urinary excretion of estrogens (47). In contrast to most mammalian species, estrogen levels gradually decline from the peak reached at day 210 of pregnancy until parturition (59, 67).

The pregnant mare is unique in that she excretes two estrogens having unsaturated B rings, namely, equilin and equilenin (45). The synthesis of these estrogens is interesting because the pathway does not involve cholesterol as an intermediate (19).

## D. FETAL GONADS

The fetal gonads of the horse (testicular or ovarian) become greatly enlarged during the latter part of gestation. The enlargement involves a proliferation of interstitial cells (46) with maximum size reached at the 6th–8th month of pregnancy. The gonads then diminish to about one-fifth this size at term (30). The causal factors associated with the enlargement of the fetal gonads have not been identified.

# IV. Parturition

## A. SIGNS

The mammary glands start to fill 3–4 weeks before parturition. Small amounts of colostrum escape through the teat orifices about 2 days before

foaling, resulting in the formation of a waxy material on the ends of the teats (waxing). Other signs of impending parturition include the development of vulvar edema, relaxation of the sacrosciatic ligaments so that the overlying muscles undulate when the mare moves, sweating, restlessness, pawing, switching the tail, and intermittent lying down and standing. These signs are all indicative that delivery is imminent (9). The mare appears to be able to exert some control over the time of day that delivery takes place as most foals are born during the night (77).

## B. DELIVERY

The actual time of delivery in the mare is very short, often accomplished within 10 or 15 minutes once the fetus enters the birth canal. The mare is able to generate considerable pressure on the foal through abdominal press (voluntary contraction of abdominal muscles with a closed epiglottis). The foal is presented through the vulva with one leg slightly ahead of the other. This presentation reduces the size of the shoulder circumference and thus facilitates delivery. The foal is often delivered while still within the amniotic membranes.

The umbilical cord of the fetus is long and most often still intact at birth. Most mares are recumbent at delivery and the foal and mare will often remain connected by the umbilical cord for 5 or 10 minutes after foaling. This relationship is important in that the foal can receive a considerable amount of blood from the fetal placenta as the uterus contracts (78). When manual delivery of a foal is being attempted, it is important to avoid rupturing the umbilical cord as the foal is pulled from the birth canal.

## C. HORMONAL EVENTS

The mare does not have a withdrawal of progesterone prior to delivery but delivers in the presence of high progesterone levels (59). Estrogen levels are maintained through the time of delivery and then decline very rapidly as would be expected since the placenta (or feto-placental unit) is the most likely source of estrogen synthesis.

The mare's peripheral blood levels of corticosteroids do not change prior to the delivery process except for a rise at delivery which is probably stress-induced. A significant rise in corticoids has been observed 15 days postpartum (59). The reason for this rise is not apparent but it may be associated with lactation.

## D. INDUCTION OF PARTURITION

### 1. Glucocorticoids

Alm, Sullivan, and First (6) successfully induced premature delivery with exogenous dexamethasone. Pregnant mares delivered prematurely (328 versus 340 days for controls) when given 100 mg on each of days 321 through 324 of gestation. Although the foals were slightly weaker when delivered prematurely with dexamethasone, all survived and grew at a normal rate. Milk production in the mares appeared to be normal. Placentas were not retained in any of the treated mares, but the levels of corticosteroids necessary for induction in the mare were higher than for other large domestic species.

### 2. Oxytocin

Oxytocin has been used to induce parturition in mares, particularly during the last few days of gestation (26, 76). Delivery occurs on an average of 35 minutes after the administration of 40 units of oxytocin (unpublished). Mares induced with oxytocin should be at term and have formed colostrum prior to induction, as there is little evidence that oxytocin alone will initiate lactation.

## V. Postpartum Period

### A. PLACENTAL RELEASE AND UTERINE INVOLUTION

Placental release usually occurs within  $\frac{1}{2}$  to 1 hour after delivery. Retention of the placenta in the mare may be serious because it is frequently associated with infection of the uterus and laminitis. Normally after expulsion the placenta is inspected for any pathological lesions and to ascertain whether it has been passed in its entirety.

The diffuse epitheliochorial type of placentation of the mare allows involution of the uterus to proceed rapidly in the postpartum period.

### B. FOAL HEAT

Follicles begin to develop soon after parturition with mares manifesting estrus and ovulation approximately 10-13 days after foaling (foal heat) (7). There is considerable pressure on stud managers to breed mares at the foal heat because of the dating age of foals and the 340-day gestation period of the mare. Mares, as a general rule, should only be bred at foal

heat if delivery, including the release and appearance of the placenta, is normal.

## VI. Reproductive Physiology of the Stallion

### A. MALE REPRODUCTIVE SYSTEM

#### 1. Testes

The testes are oval in shape (7.5–12.5 cm long  $\times$  4–7 cm dorsoventrad  $\times$  5 cm wide) with a slight compression from side to side. The long axis of the unretracted testicle is almost horizontal with the head of the epididymis in an anterior position, the body dorsal to the testis, and the tail in a posterior position (77).

The testes produce androgens and estrogens. Peripheral blood levels of testosterone, presumably produced by the Leydig cells, has been reported to average 1.9 ng/ml of plasma in one study (15), and 0.07–1.6 ng/ml in another (33). Cryptorchid testes continue to secrete androgens (38). Equine testes appear to secrete estrogens, as high levels have been reported in testicular tissue (61, 106). The source of estrogen synthesis within the testis, reputed to be the Sertoli cell, remains unknown.

Males are occasionally encountered that have neither visible nor palpable testes. Animals with testicular tissue within the abdominal cavity respond to preparations with LH activity (HCG) by a rise in testosterone concentration within 30 minutes after administration. Geldings (castrated males) can be identified because they do not respond to HCG administration (33).

#### 2. Accessory Sex Organs

pulla The secretion of the seminal vesicles, gelatinous in texture, has a high concentration of citric and lactic acids (61, 103)

The prostate, a lobulated structure situated on the floor of the pelvis above the neck of the bladder, can with some difficulty be palpated per rectum It is about 5 cm long and 2.5 cm wide The paired bulbourethral glands (Cowper's glands) can be found near the ischial arch

An assessment of the functional state of organs such as the prostate, seminal vesicles, and epididymides can be done through the chemical determination of such substances as glycerylphosphorylcholine, citric acid, inositol, and ergothioneine in the ejaculated semen, or in the secretion of the accessory glands directly The reader is advised to review the work of Mann (61) and White and MacLeod (103) for detailed information on the composition and biochemistry of semen

## B REPRODUCTIVE ACTIVITY

Although sexual activity may be influenced to some extent by the season of the year, stallions will breed at any time of the year The stallion should have a general physical examination for breeding soundness prior to the breeding season and should be observed in the presence of a mare in estrus for premounting libido, erection of the penis, positioning for mounting the mare, mounting, exploratory movements with the penis for the vulva, copulatory movements, 'flagging' (rhythmic contractions of the tail accompanying ejaculation), and ejaculation, relaxation of the penis, dismounting, and shrinkage of the glans (22) The main abnormalities that may be found are lack of libido, failure of erection, or failure of ejaculation, the presence of pain in connection with the breeding process is also of clinical importance

Although conditioned stallions often become aroused with the first preparatory movements to bring them out of the stall, sexual excitement usually begins when the stallion first sees the mare Complete erection should be attained prior to allowing the stallion to mount the mare Erection time has been reported to range from 119 to 163 seconds and time for the mounting reflex from 101 to 206 seconds (105) Pressure within the corpus cavernosum penis was found to be 13 mm Hg during relaxation of the penis with an increase to 107 mm Hg upon first arousal, and finally to 6530 mm Hg during coitus (10)

Intromission is usually achieved following several copulatory movements This is followed by ejaculation in about 13 seconds (105) An average of 1.4 mountings per ejaculation was observed with natural mating while 2.2 mountings per ejaculation were required when an artificial vagina was used to collect semen (21, 105)

The ejaculation pattern has been demonstrated through the studies of Tischner, Kosiniak, and Bielański (95). Emission occurred after an average of seven (5-11) intravaginal thrusts in the form of 8 (5-10) seminal jets. The first three jets contained 80% of the ejaculated spermatozoa and were emitted under high pressure. The subsequent jets were emitted under lower pressure, and declining erection as the penis was withdrawing.

### C. SPERMATOGENESIS

Sexually mature stallions have well-defined associations of spermatogenic cells which succeed one another in a cyclic pattern (64, 93). The mean duration of one cycle of the seminiferous epithelium is 12.2 days (93). The duration of the entire process of spermatogenesis extends over four consecutive cycles of the seminiferous epithelium and thus is 49 days in duration (93). Epididymal transit time is approximately 5 days in the stallion and thus the time required to develop spermatozoa to the point of ejaculation is 54 days (93). This is an important consideration when semen evaluation results are poor and a reevaluation is required.

Spermatozoa from the testes undergo maturation during their passage through the epididymis. The spermatozoa of some species undergo further physiological change during transit within the female reproductive tract before the capability to fertilize ("capacitation") is achieved. The question as to the necessity for capacitation of stallion spermatozoa remains to be answered.

The presence of large numbers (12,000/ml) of immature spermatozoa (primary and secondary spermatocytes and spermatids) has been reported in the semen of some mature stallions (92). It has been suggested that focal testicular degenerative lesions are responsible for the presence of immature germ cells in the semen. The cause of the lesion is unknown, but several possibilities include (1) local bacterial infection, (2) thermal degeneration, (3) nutritional deficiencies, and (4) chemicals, drugs, or ionizing radiation. Although stallions affected with this syndrome are fertile, their fertility rate may be lower than normal (92).

### D. SEMEN EVALUATION

There is no single test of semen which can serve as an absolute indicator of fertility for the stallion. Table I presents some semen characteristics which are usually determined in assessing the potential fertility of a stallion. Most fertile stallions have (1) sperm in average to high numbers, (2) semen with a high percentage of sperm with progressive motility, (3) semen with a low proportion of dead sperm, (4) sperm with a low proportion

**TABLE I**  
**Semen Values for Fertile Stallions<sup>a</sup>**

	Average	Range
1. Volume (ml)		
Total	70	30-300
Gel-free	58	45-76
Gel	27	0-200+
2. Concentration (millions/ml)	120	30-800
Gel-free	282	222-321
3. Total sperm (billions)	8.4	4-20+
4. Percentage progressively motile	73	60-95+
5. Percentage morphologically normal	75	65-94
6. pH	7.4	6.8-7.8

<sup>a</sup> From references (18, 22, 77, 105).

of abnormal forms (primary <10% and secondary <30%), and (5) a survival time over 24 hours when diluted with a 7% glucose extender and held at 0° to +4°C (17, 21, 22, 57). In one study, the concentration of semen, volume of semen, percentage spermatozoa morphologically normal, and the initial progressive motility after washing and extending the semen were the factors which best explained the variation in the number of mares bred and diagnosed pregnant by any one stallion (57).

Evaluation of stallion semen must take into consideration season of the year, age, breed, condition, sexual activity, and state of health of the animal. The initial examination of a stallion should be conducted after a week of sexual rest. The condom and the artificial vagina are the two most common methods used for semen collection (68, 73, 77). The ejaculates vary in color from milky-white to watery-gray while the consistency is usually gelatinous. The stallion is usually collected twice at a 1-hour interval. While the volume of the two ejaculates should be approximately equal, the second ejaculate usually contains about one-half of the number of spermatozoa found in the first (74). The gel is removed by a filter before evaluation of the remaining portion of the sample.

Sperm production is influenced by the season of the year, size of the testicle, frequency of ejaculation, and the age of the animal (22, 57, 61, 68, 74). Total sperm numbers in the fall and winter are approximately one-half the number found in the spring and early summer (74). Frequency of semen collection, while not affecting seminal volume, can affect sperm numbers (22). Stallions collected once per week or every other day had approximately the same number of spermatozoa per volume, but when they were collected six times per week the sperm numbers dropped significantly



(74). Daily collection for 6 days is required to deplete extragonadal sperm reserves. The number of spermatozoa obtained on the seventh consecutive daily collection should approximate the daily sperm output (39). Stallions with small testes will not have the sperm-producing potential of normal stallions with larger testes (74).

Many stallions with normal fertility harbor bacteria in their genital tracts without producing recognizable disease (49). These stallions are a potential source of bacterial contamination to susceptible mares. Although certain strains of bacteria may affect sperm survival or cause the establishment of infection and thus affect fertility, most mares are unaffected by the bacteria to which they are exposed when bred. A stallion should not be considered unfit for breeding only because bacteria are cultured from the semen or urethra (53).

## E. ARTIFICIAL INSEMINATION

Artificial insemination (AI) for domestic animals was first used successfully in the mare (25). Although AI is a simple procedure, it still has very limited use in most parts of the world. AI in the mare is practiced most commonly in China, Russia, and Japan (25). It is used most often in the United States on Standardbreds, Quarter horses, and some Arabians.

Artificial insemination of mares with raw semen, or semen extended in skim milk or cream gelatin diluters at the correct time during estrus will result in conception rates that are as good as obtained in natural breeding (25, 52, 74). A number of reports have appeared since 1956 on the successful freezing of stallion semen in pellets or vinyl straws with storage carried out in liquid nitrogen ( $-196^{\circ}\text{C}$ ) (58, 62, 68, 101).

# VII. Genetic Aspects of Reproduction

## A. NORMAL CHROMOSOME COMPLEMENT

Excellent karyograms have been obtained by using lymphocytes which have been purified by density gradient procedures before culturing (94). Cells undergoing division are then arrested, swollen by hypotonic solutions, fixed, and stained prior to being arranged in pairs according to their morphological features. The normal chromosome complement of the horse is 64, the donkey 62, and the mule and hinny 63 (12).

## B. EQUINE HYBRIDS

The most common equine hybrid is the mule (donkey male  $\times$  horse female). The reciprocal cross is called a hinny (horse male  $\times$  donkey fe-

male). Zebras can also be crossed with horses or donkeys to produce hybrids. Hybrid offspring have chromosome numbers intermediate between those of their two parents (12, 82). The sterility of male hybrids is due to a block in spermatogenesis during meiosis because maternal and paternal chromosomes are dissimilar in size and number, making true pairing impossible. Thus while the testes of male hybrids appear to secrete testosterone, they do not form spermatozoa (82). Female mules have been observed to come into estrus and ovulate on a somewhat irregular basis (20). Whether these ovulations are ever fertile is highly speculative (12, 13, 20, 82). Chromosomes of an alleged mule who foaled were like those of a donkey, in spite of some mulelike characteristics of the animal (13).

### C. CHROMOSOMAL ERRORS

#### 1. Gonadal Dysgenesis (Turner's Syndrome)

This condition has been recognized in mares in which small, inactive ovaries are present (Fig. 11). The ovaries lack germ cells and consist pri-



FIG 11. Small, firm, and inactive ovaries from a mare with a chromosome karyotype of 63,XO showing absence of follicular development. The cut surface on the left exposes a band of pigment containing cells (From Hughes *et al*, 50)

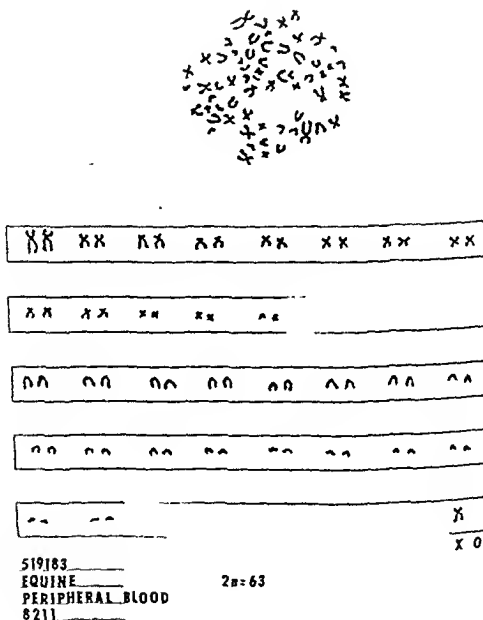


FIG. 12. Karyotype of chromosomes from a dividing lymphocyte from a mare showing 63,XO. A spread of chromosomes is shown at the top of the figure and the cut-out chromosomes are arranged in pairs cut from a photographic print at the bottom. A single X chromosome is shown in the lower right-hand corner. (Courtesy Trommenshausen-Smith and Hughes.)

marily of undifferentiated ovarian stroma. The tubular genitalia are small. Karyotypes of the mares are mainly 63,XO (Fig. 12) with occasional mosaics of 64,XX/63,XO reported (50, 51). An infertile mare with a chromosome count of 63 due to an apparent lack of an autosome has been reported in which follicular development and ovulation occurred (50, 51).

## 2. Intersexuality

Intersex animals are those that possess anatomical characteristics of both sexes. These are divided into three main classes: "true" hermaphrodites, male pseudohermaphrodites, and female pseudohermaphrodites. True hermaphrodites, i.e., animals having both ovarian and testicular tissue, are very rare in horses.

Pseudohermaphrodites have the gonads of one sex, but the external genitalia and other characteristics resemble those of the opposite sex. The male pseudohermaphrodite is the most common intersex in horses and characteristically has hypoplastic testicles within the abdominal cavity or inguinal canal, a penislike clitoris emerging from a rudimentary vulva, and exhibits male libido (24, 40).

The karyotype of most male pseudohermaphrodites is that of the normal female ( $2n = 64,XX$ ) (24, 35). Several other karyotypes have been described having the following chromosome patterns: 64,XX/64,XY; 66,XXXXY; 64,XX/65,XXY; 64,XX/64,XY/65,XXY. The 66,XXXXY and the 64,XX/65,XXY chromosome patterns resemble some of the karyotypes noted for Klinefelter's syndrome in man (24, 35).

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## II. Cycles in Reproduction

### A. THE LIFE CYCLE

There are four major periods in the life cycle, namely, fetal, prepubertal, reproductive, and senescent. Reproductive efficiency reaches a peak early in the reproductive period, remains high for several years, and declines thereafter.

#### 1. The Fetal Period

The origin, migration, and differentiation of the primordial germ cells are discussed in Chapter 7 and the details of their development may be found in the following references (23, 24, 51, 67). The chronological development of the genital organs in the bovine fetus is shown in Table I.

TABLE I  
Sexual Differentiation of the Calf Fetus\*

Age (days)	Crown-rump length (mm)	Males	Females
39-40		Early testicular organogenesis	Undifferentiated gonadal primordium
42-43	24-26	Definite albuginea	
45	29	Testicular interstitial cells	
47	32	First flexure of penile urethra	
48-49	34-36	Anogenital distance definitely increased	Beginning of prolonged and slow thickening of the superficial ovarian layers
		Growing Mullerian ducts in both sexes	
50-52	38-42	Upper Mullerian ducts: reduction in diameter	
56	53	Prostatic buds and seminal vesicles appear	
		Male urogenital connections	
58-60	59-66	Mullerian ducts: anterior part disappears	Uterus increases in diameter
		Penis opens under the umbilicus	
		Balanopreputial fold in organization	
		Scrotum develops	
70	115	Mullerian ducts absent or regressing	Retrogression of Wolffian ducts begins
		Formation of epididymis	
75	—		First premeiotic figures

\* From reference (52).

## 2. The Prepubertal Period

At birth, the primary oocytes, some 75 to 160 ( $\times 10^3$ ), are arranged around the periphery of the ovary (24, 46). Tertiary follicles with antra appear late in fetal life (66) and their number increases after birth, reaching a maximum at the end of the second month (24). Prior to puberty the ovaries, despite the presence of follicles with antra, are small, and the onset of ovulatory cyclic activity is a gradual process preceded by anovulatory and otherwise incomplete cycles. Thus luteinizing hormone (LH) is detectable in blood plasma at normal adult basal levels (0.5 to 2.0 ng/ml) from birth, with peaks up to 30 ng/ml detectable as early as 6 weeks (85), and continuing to puberty at 41 to 60 weeks. Although of normal post-pubertal magnitude, these LH peaks do not induce ovulation. When such ovulations are induced, corpora lutea develop normally but in many instances they persist, indicating that the prepubertal tract may lack the ability to initiate leutolysis (93).

In the bull calf, the approach of puberty and the production of viable spermatozoa are associated with the size of the testicles, for which there are three distinct phases of growth (5) characterized by a progressive development of the spermatogenic cycle (Table II). These changes are not associated with any dramatic changes in pituitary LH output, but there is a relationship with androgen (60).

## 3. Puberty

Puberty in the heifer is a much more dramatic event than in the bull. There is a great increase in ovarian size, due to the secretion of liquor

**TABLE II**  
**Development of Spermatogenic Activity in the Bull Calf\***

Phase of testicular growth	Live weight (kg)	Types of cell present in sex cords	
1	30-100	Supporting cells	Gonocytes
2	100-300	Supporting cells	Spermatogonia
			Spermatocytes
			Spermatids
3	300-800	Sertoli cells	Spermatozoa

\* From reference (72).

# 16

## Reproduction in Cattle

T. J. Robinson

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### I. Introduction

Asdell has summarized the basic data concerning reproductive phenomena in cattle (4). Most domesticated breeds of cattle breed all the year round but there is a seasonality of breeding efficiency. As with other mammals, three cycles are involved in the processes of reproduction of each sex, namely, the life cycle, the annual breeding cycle and, in the female the estrous cycle and, in the male the spermatogenic cycle.

## II. Cycles in Reproduction

### A. THE LIFE CYCLE

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2	100-300	Supporting cells	Spermatogonia
			Spermatocytes
			Spermatids
3	300-800	Sertoli cells	Spermatozoa

\* From reference (72).

folliculi, associated with an ability of follicles to ovulate as a consequence of an LH peak. In the 2 months preceding puberty no marked changes are evident in serum follicle stimulating hormone (FSH), prolactin, or gonadotropin-releasing hormone (GnRH). Levels of estradiol are relatively high prior to day 42 and then fall to base levels while LH levels fluctuate widely. The data of Gonzalez-Padilla *et al.* (28) suggest that one and possibly two "silent" cycles precede first estrus (day 0). The data, based upon daily pooled serum samples, provide no evidence of an estrogen peak preceding that of LH and estrous behavior, and the authors conclude that progesterone plays a key role in the changes leading to the establishment of phasic LH release. Be this as it may, these heifers did exhibit estrus, suggesting that the necessity to pool samples precluded the possibility of detecting a transient estradiol peak at the very low level required for a physiological effect (12).

The time of onset of puberty appears to be a function more of "size" than of age, but it is difficult to define "size" so that dimensional measurements—height at withers and body length—seem more useful parameters than live weight (92). Under good nutritional conditions a calf attains puberty at approximately two-thirds adult "size." Restricted nutrition, even of a relatively minor nature, delays puberty (40). Joubert (53) has attempted to separate the age and weight components affecting the onset of breeding activity (Table III).

In the developing male, undernutrition results in a delay in the onset of puberty due to a marked impairment of testicular development and sperm production, associated with inhibition of androgenic function and consequent fructose and citric acid production by the seminal vesicles (19, 61).

TABLE III

Mean Ages and Weights at Puberty of Four Breeds of Cattle Reared on a High or Low Plane of Nutrition\*

Plane of nutrition during rearing	Mean weight at first estrus (lb)	Mean age at first estrus (days)
High	530.7 $\pm$ 40.0	440 $\pm$ 35.1
Low	579.4 $\pm$ 41.6	710 $\pm$ 62.1

\* From reference (53).

#### 4. The Reproductive Period

Once breeding activity commences, it can continue for many years. The number of primordial follicles remains static until about the fourth year (mean 133,000) and declines thereafter until near zero between 15 and 20 years (24).

Testicular size is a good index of sperm production and reproductive activity. Efficiency of sperm production per gram of testicular tissue reaches a maximum at 11 months of age and thereafter remains constant. Total production, however, continues to increase as a result of the doubling of the weight of the testes over the next 5 or 6 years (Table IV).

#### 5. Senescence

This is of little practical interest as few cattle (some 0.25%) normally are kept to 15 years of age.

#### B. THE ANNUAL BREEDING CYCLE

Even in temperate climates there is a strong tendency for cows to calve in the spring. Hours of daylight have been implicated as an important factor controlling this seasonality in *Bos taurus* (95) but other factors such

TABLE IV  
Development of Sperm Production in Holstein Bulls\* b

Age	No. bulls	Gross weight paired testes (gm)	Daily sperm production	
			10 <sup>6</sup> /bull	10 <sup>6</sup> /gm testis
0-4 mo	25	20	0	0
5-7 mo	15	97	104 <sup>c</sup>	1 <sup>c</sup>
8-10 mo	20	284	1750	7
11-12 mo	15	370	3300	10
17 mo	13	480	4480	10
3 yr	10	586	6040	11
4-5 yr	11	647	6530	11
>7 yr	11	806	8000	11

\* From reference (1)

<sup>b</sup> Daily sperm production was calculated from testicular homogenate counts using a time divisor of 5.32 days.

<sup>c</sup> Mean for six bulls producing spermatids or spermatozoa.

as temperature and nutrition are involved. Bulls also show a seasonal variation in fertility in which both photoperiod and temperature are involved; long days favor sperm production; high temperatures depress semen quality (14, 70).

Photoperiod has also been implicated in seasonality of breeding of Zebu cattle (*Bos indicus*) (21) and buffalo (*Bubalis bubalis*) (71) but other correlated environmental factors, e.g., temperature, rainfall, and feed supply, appear to be of overriding importance (50). Nevertheless, a fundamental effect of photoperiod appeared operative in Zebu cattle studied in the Mexican Gulf coast region. Pubertal heifers had a high rate of conception during the unfavorable dry season, to provide an early spring calving. However, with successive calvings, the tendency to conceptions in the rainy season increased (Table V), so that by the fifth calving almost the entire herd was relieved of this fundamental constraint. This principle applies also to *Bos taurus*, reared in tropical environments (25), and to the African buffalo (32).

This overriding influence of rainfall, and consequent pasture growth and plane of nutrition, appears due to a necessity for a buildup in body condition prior to conception (55) and is as important in improved dairy herds as in primitive buffalo.

Social factors may have a further modifying effect. Thus Skinner and Bonsma (90) observed that the mean time to day of mating after introduction of a bull to cows run alone was 19.3 days as compared with 9.5 days for cows run with a vasectomized bull.

TABLE V  
Order of Consecutive Conceptions in Zebu Cows and Their  
Distribution in Relation to the Dry and Rainy Season\*

Conception number	Number and percentage conception				Total No.
	Dry season January-May		Rainy season June-October		
	No.	%	No.	%	
1	190	48.6	201	51.4	391
2-4	245	36.2	431	63.8	676
5-7	139	27.5	366	72.5	505
8-10	78	27.9	202	72.1	280
	652	35.2	1200	64.8	1852

\* From reference (50).



### C. THE ESTROUS CYCLE

The characteristics of the estrous cycle of domestic and other cattle have been discussed in detail for the cow in the earlier editions of this work (18, 37). The length of the cycle is 20 days in heifers (S.D. 2.3 days) and 21 days in cows (S.D. 3.7 days). Estrus is of short duration, 12–24 hours, but the range is considerable. For convenience, the day of estrus is commonly designated day 0. Ovulation, which normally occurs after the end of estrus, is spontaneous.

Events preceding the first ovulation at puberty are now being studied (28). Once initiated, the events follow an orderly, well-timed sequence. Following ovulation a "corpus luteum" forms under the influence of pituitary luteotrophin and secretes progesterone for a finite time. Both LH and prolactin have been implicated as the luteotrophin but there is still controversy concerning the importance of prolactin and whether or not estrogen is involved. Estrogen is produced in small quantities during the luteal phase, with a minor peak between 7 and 11 days (27, 42, 86) associated with the development and subsequent regression of a mid-cycle follicle (78). In the presence of a functional corpus luteum, this peak of estrogen is unable to initiate the release of LH (44). Degenerative changes in the corpus luteum are apparent by the 18th day (36), associated with a decline in systemic blood levels of progesterone (79). There follows an increase in level of systemic estrogen, mainly estradiol-17 $\beta$  (22), which reaches a peak before the onset of estrus (42, 86). Unlike the situation in the sheep, it is not clear in the cow whether or not the prostaglandins, notably prostaglandin F<sub>2 $\alpha$</sub> , are actively involved in the process of luteolysis. An increase in the level of endometrial prostaglandin F<sub>2 $\alpha$</sub>  coincident with the demise of the corpus luteum has not been conclusively demonstrated although it is well established that exogenous prostaglandin, intrauterine or systemic, is highly effective as a luteolytic agent (84). Relieved from the constraints of a high level of circulating progesterone, the ensuing high level of circulating estrogen initiates the release of gonadotropin (17, 44). This, seemingly, is accomplished by the stimulation of the release of of decapeptide gonadotropin-releasing hormone from the hypothalamus which, when transported by the hypophyseal portal system to the hypophysis, rapidly induces release of FSH and LH (105).

These events, reviewed by Denamur (20) and illustrated in Fig. 1, pose a basic question concerning the fundamental nature of the estrous cycle. Is there an intrinsic pattern of growth and regression of follicles, with associated production of estrogen, which constitutes a biological "clock" and is this responsible for the accurate timing of the cycle, as in the laboratory rodents? The estrous cycle of the ruminants bears a remarkable resem-

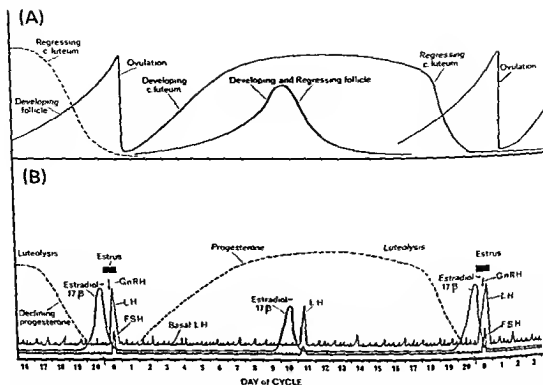


FIG. 1. Morphological (A) and endocrinological (B) events in the estrous cycle of the cow.

blance to the pseudopregnancy of the rat and mouse and, in a biological sense, the cycle may be regarded as a pseudopregnancy which overrides, or is geared to, a fundamental cycle of growth and regression of follicles with an associated peak in estrogen production about every 10 days in the cow and every 4 days in the sheep.

The physiological changes in the reproductive tract consequent upon these endocrinological events have been described by Asdell (4) and Hansel (37). Estrus is characterized by anatomical and behavioral changes described in Chapter 10. Problems posed by "silent heats" and heats of short duration are not uncommon (18) and may be exacerbated, especially on the range, by malnutrition (55) and stress (99). Further, the time of estrus during the day is not random in *Bos taurus* (97) or *Bos indicus* (47). The impact of social and environmental factors on the efficiency of detection of estrus and of mating in cattle has not been studied as intensively as in the sheep. Bleeding occurs from the uterus in many animals during metestrus, usually about 24 hours after ovulation.

#### D. THE SPERMATOGENIC CYCLE

The liberation of spermatozoa by the testes of the bull is a continuous process, some 10 to 13 ( $\times 10^6$ ) per day being produced by an adult (1)

of which about one-half can be harvested in successive daily collections (35). This process is the result of an accurately timed spermatogenic cycle of some 60 days duration (16).

### III. Mating and Fertilization

Mating behavior is a direct effect of estrogen but, as in the sheep, progesterone is involved. The heifer is highly sensitive to estrogen, the median effective dose (MED) of estradiol benzoate (EB) required to elicit a full estrous response being some 120–130  $\mu\text{g}$  (Table VI).

Although ovulation occurs spontaneously, it may be hastened by mating (63, 77) and there is some evidence for a circadian rhythm (48). Normally, one egg is shed, with the right ovary ovulating more frequently than the left (4). The incidence of twin ovulations ranges from about 2% for dairy cattle declining to 0.5% for beef breeds.

The ovum normally is shed some 10 hours after the termination of estrus. In the next 6 hours it travels about one-third of the way down the oviduct during which time it is fertilized, some 30 hours after the onset or 20–24 hours after the middle of estrus. Early reports on conception rates following artificial insemination show maximum fertility to insemination at mid-estrus, some 13–18 hours before ovulation (97). At first sight this is rather puzzling in view of reports of rapid transport of spermatozoa to the site of fertilization (98).

Recent studies on sperm transport in the female reproductive tract shed some light on this apparent mystery (34). Hafez (33) has postulated three phases of sperm transport, namely, (a) a rapid passive phase during which

TABLE VI  
Response of Spayed Heifers\* to Estradiol Benzoate (EB) with and without Pretreatment<sup>b</sup> with Progesterone<sup>c</sup>

Dose of EB ( $\mu\text{g}$ )	EB after progesterone				EB alone			
	No. of heifers		Mean time to onset (hours)	Mean duration (hours)	No. of heifers		Mean time to onset (hours)	Mean duration (hours)
	Treated	Estrus			Treated	Estrus		
400	14	13	19.4	5.8	14	1	21.0	3.0
200	14	14	19.7	8.6	14	2	27.0	3.0
141	14	9	25.0	4.3	14	4	26.2	7.5
100	14	4	25.5	5.2	14	1	27.0	3.0

\* Previously made refractory to estrogen by repeated injections.

<sup>b</sup> 10 mg/day for 5 days followed 3 days later by EB.

<sup>c</sup> From reference (12).

some spermatozoa may be transferred rapidly to distal parts of the female reproductive tract, (b) a phase of colonization of reservoirs during which the spermatozoa invade the protective cervical crypts, and (c) a slow phase of transport during which spermatozoa are released from the primary reservoir (the cervix in the case of the cow) and progress to and through the uterus and to the site of fertilization in the upper third of the Fallopian tube. There is little evidence that the first phase is important. Rather there appears to be a gradual buildup at the uterotubal junction, which region acts as a protective reservoir while the isthmus acts as a filter (34). The cervix is the primary reservoir (64) and from it there is continuous replenishment of the uterotubal junction for some 3 days.

Failure of fertilization in cattle exhibiting normal estrus is not a major problem. Nor, by contrast to the sheep, does it appear to be a problem in cattle ingesting phytoestrogens. It is a problem, however, in cattle in which estrus has been synchronized by treatment with exogenous progestagen (34, 68). Although direct evidence is lacking for the cow it seems certain, by analogy with the ewe, that this is due to impairment of the normal pattern of sperm transport. Despite the considerable literature on nutrition and fertility in cattle (55), definitions are lacking of the nature of the subfertility associated with undernutrition and with specific nutritional deficiencies (e.g., phosphorus). Failure of fertilization due to an impairment of normal sperm transport and survival is an obvious area for investigation. Finally, there are genetic defects which prevent fertilization and which constitute an important but not primary cause of subfertility (94).

#### IV. Pregnancy

Despite the high percentage of healthy breeders in which eggs are fertilized following insemination (90–100%) only approximately 50 to 55% calve (Table VII). The progressive loss is due to embryonic and fetal death (7), the former being much the greater.

The establishment and maintenance of a successful pregnancy involves three periods, that of the ovum, the embryo, and the fetus. During the first, the fertilized dividing ovum, within its *zona pellucida*, passes down the fallopian tube to the uterus. During the second, differentiation occurs and pregnancy is established. During the third, the placenta is fully developed and the fetus makes rapid growth.

##### A. THE PERIOD OF THE OVUM

The fertilized egg enters the uterus at between 72 and 96 hours, at the sixteen-cell stage. This timing is critical (8, 83) since survival is dependent

TABLE VII

Percentage of Cows Pregnant at Various Stages as Estimated by Pregnancy Diagnosis or Nonreturn to Service<sup>a</sup>

Interval from service				
1 month	2 months	3 months	9 months	Calving
67.8	58.4	55.7	—	52.9
74.9	68.3	65.3	58.6	53.2
78.4	—	65.9	—	53.5

<sup>a</sup> From reference (81).

upon the uterus being under the influence of progesterone produced by the developing corpus luteum.

## B. THE PERIOD OF THE EMBRYO

A series of coordinated events is involved. First, there is division of the zygote followed by differentiation into embryonal and placental elements, evident by the eighth day, about which time the zona pellucida is lost. Second, there is elongation of the blastocyst, often referred to as the chorionic sac, which by the 18th day fills the uterine horn in which the fertilized ovum lodged and may grow into the opposite horn. Third, there is a gradual erosion of the epithelium of the caruncles by the trophoblast, followed by an active invasion by trophoblast cells which leads to the development of the fetal/maternal cotyledonary placenta (Fig. 2). Implantation is a gradual process, characterized by a progressive invasion of the caruncles which is not completed until the end of the first third of pregnancy (2, 10).

This first six weeks of pregnancy is a period highly susceptible to loss (see Table VII) with, on the average, about 31 to 34% of fertilized eggs failing to survive (81). No single factor can be implicated, nor is it possible to apportion the loss between genetic causes and failure of the maternal environment. The latter is important in the cow, as shown by egg transfer studies (8, 83). A two-day asynchrony between the endocrine state of the uterus and the stage of development of the fertilized egg results in embryonic death. Proximity to another embryo also reduces the chances of survival as shown by a doubling of the chance of twins when one egg is shed from each ovary as compared with both from one (31) or when one is transferred to each uterine horn (82).

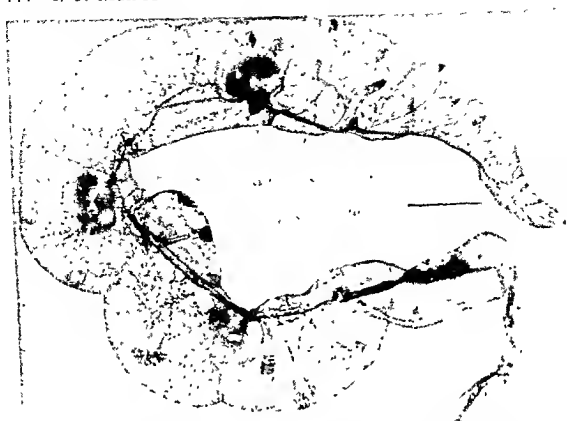


FIG. 2. Fused allantochorion in a triplet set, 49 days after insemination showing fetal components of the cotyledons and the three fetuses contained in their individual amnions. Anastomoses of both large and small blood vessels are visible. The fetus in the middle was a female, the others were males. Scale, 5-cm large squares seen on the graduated paper. From Jost *et al.* (52), courtesy Blackwell Scientific Publications.

In the case of such multiple pregnancies, adjacent trophoblasts (chorions) fuse and when the allantois, with its associated blood vessels, grows out to invest the chorion to provide the allantochorion there is early anastomosis of the blood vessels.

### C. THE PERIOD OF THE FETUS

Differentiation into the component parts of the new organism and its membranes is effected by the end of the sixth week. Thereafter pregnancy consists of consolidation and growth of the placenta, which does not attain its mature form until the third or fourth month (36), and growth of the fetus. The development of the epitheliochorial placenta (69) is elegantly described and illustrated by Amoroso (2) and more briefly by Harvey (41).

During the first two-thirds of pregnancy, the rate of growth of the placenta exceeds that of the fetus, the growth of which is so remarkably

uniform that fetal age can be determined by reference to standard measurements and photographs (102). During the last one-third, the situation is reversed, and it is during this period that nutritional stresses affecting birth weight and survivability are important.

#### D. FREEMARTINS

In 1917, Lillie (58) postulated that the changes wrought in heifers born twin to a bull were due to internal secretions borne to the heifer by an anastomosed placental circulation. In recent years this freemartin syndrome has formed the central theme of a symposium on intersexuality (74), and has been the subject of review (62) and discussion (52, 87, 88).

The "freemartin" condition (free = sterile; martin = bovine) is a direct result of the form of placentation in the cow. In multiparous ruminants, adjacent trophoblasts fuse to form a common chorion. In some, such as the sheep, the blood vessels which grow out with the allantois of each embryo in the formation of the allantochorion appear not to come into close juxtaposition with those of the adjacent embryo. In the cow, by contrast, they do. They occupy the same cotyledons, and there is fusion of placental blood vessels (Fig. 2), well-established by the 39th day (52). This permits a degree of exchange of blood between fetuses, with consequent profound effects, in 90% of cases.

In the fetal calf the testes of the normal male become histologically recognizable about the 40th day whereas the female gonads remain largely undifferentiated for several weeks longer (see Table I). Disturbance in development is first apparent at about the 50th day when the Mullerian ducts of males and of future freemartins commence to decrease in diameter relative to those of normal females (Fig. 3); by the 62nd day they are vestigial (52). Over the same period there is inhibition of growth of the presumptive ovaries.

This time difference between male and female organogenesis has led Jost *et al.* (52) to suggest that the destruction of the Mullerian ducts is caused by an "antifeminine" substance or hormone (not testosterone) produced by the male twin. There then follows a positive masculinization process caused by a second testicular factor which stimulates the vestigial ovaries, seminal vesicles, and other structures, resulting in a range of conditions including, in some cases, an ovotestis which later takes over an endocrine function. In no case are the external genitalia greatly modified, so that the freemartin remains phenotypically female.

Not only hormones are exchanged between fetuses. Cellular and other elements normally in blood are transported, and there is the possibility that these include cells from the gonads and other organs. The lodgement





in the female co-twin of gonadal cells responding in a malelike fashion to tropic hormones could be implicated in the basic changes (43) and would also account for increasing masculinization with age

The majority of cattle twins have identical blood types (62) indicating that cells capable of being established in hematopoietic tissues of co-twins are exchanged and continue to provide a source of blood cells throughout life. Further, skin grafts between nonidentical cattle twins are so well-tolerated as to be useless as a test for monozygosity in cattle twins. Females which show tolerance to skin grafts from male co-twins are freemartins, the 10% which do not are fertile (9). Sex chromosome chimerism has also been demonstrated in leukocytes, germ cells, and numerous other cell types (62).

The steroids present in the gonad, testosterone, progesterone, androstenedione, conform to the concept of a functional ovotestis (89).

Methods of diagnosis for the freemartin include clinical examination of the reproductive organs, blood typing, skin grafting, sex chromatin detection, or determination of the presence or absence of XY cells in leukocytes (62).

## V. Parturition and Lactation

These important processes of reproduction are considered in Chapters 13 and 14.

## VI. Control of Fertility

Several reproductive phenomena are amenable to a degree of artificial control. These include the time of ovulation and estrus, the number of

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FIG. 3. Dissections showing the gonads, the genital ducts, and the external genitalia of calf fetuses ( $\times 3.3$  for all fetuses). Upper row: 52-day-old fetuses. On the left, normal male; notice that the testes are less elongated than the ovaries in the female and that the penis has already moved beyond the genital swellings. In the middle, normal female. On the right, freemartin with stunted ovaries. Middle row: 60-day-old fetuses. On the left, normal male; notice the scrotum and the long anogenital distance in the middle normal female. On the right, freemartin (FM) whose ovaries show the average degree of inhibition. Lower row: Variations in 60- or 61-day-old fetuses. On the left side, normal 61-day-old female from a male female twin pregnancy with no fusion (NF) of the chorions and no vascular connections. In the middle, 60-day-old freemartin showing slight ovarian inhibition; it belonged to a quadruplet (1♂ + 3♀) pregnancy. On the right side, 61-day-old freemartin showing a severe degree of ovarian inhibition; it belonged to a triplet (1♂ + 2♀) pregnancy. From Josl *et al.* (52), courtesy Blackwell Scientific Publications.

eggs shed, and the time of parturition. In addition, a number of manipulative procedures such as egg transfer, artificial insemination, and the storage of frozen embryos are used in association with this control.

## A. SYNCHRONIZATION OF ESTRUS

Essentially there are two approaches, namely, artificial prolongation of diestrus using exogenous progestagen, or its shortening by use of a luteolysin (68).

### 1. Progestagens

Progesterone and a number of synthetic analogs have been extensively tested when injected, fed, implanted, or inserted intravaginally either alone or in combination with estrogen, gonadotropin or, more recently, prostaglandin. The early experiments with injected progesterone, reviewed by Anderson *et al.* (3), showed that although the time of ovulation and estrus could be controlled quite accurately, fertility was almost invariably low. Attempts to reduce the number of injections by using long-acting forms (e.g., starch suspensions) were unsatisfactory. Hansel (38) concluded that satisfactory synchronization could be achieved by feeding either MAP (Upjohn; 17 $\alpha$ -acetoxy-6 $\alpha$ -methylpregn-4-ene-3,20-dione) at a level of 180 to 200 mg/animal/day or CAP (Lilly; 17 $\alpha$ -acetoxy-6-chloropregn-4,6-diene-3,20-dione) at 10 mg/day for 18 days to either dairy or beef cattle. The drug could be incorporated either into a liquid ration or one pelleted or ground. Conception rates at the first estrus after withdrawal of progestagen were variable but were normal at the next cycle.

There is now a substantial literature describing many tests, almost all of a trial and error nature, on the efficacy of oral progestagens, notably MAP, CAP, and MGA (Upjohn; 17 $\alpha$ -acetoxy-6-methyl-16-methylene-4,6-pregnadione-3,20-dione) (49). The most useful practical advance has been application of the luteolytic effect of estrogen early in the cycle, thus permitting a 9-day period of progestagen feeding associated with a single injection of estrogen on day 2 of treatment (101), thus reducing costs and improving conception rates. However the time of estrus is spread over several days so that insemination at a fixed time is not feasible. Estrus is "grouped" rather than synchronized.

Recent development of synthetic progestagens of extremely high potency such as 17 $\alpha$ -acetoxy-11 $\beta$ -methyl-9-nor-preg-4-ene-20-dione (SC-21009) has made possible the use of removable subcutaneous ear implants (100, 104). Modifications include the use of PMSG (13) and PGF $_{2\alpha}$  (103) at the time of removal, each of which appears to improve fertility. The ef-

fects of several treatment parameters are shown in Table VIII. The time of estrus and ovulation relative to that of withdrawal of treatment is much more precise than that following oral treatment, and this precision may be sufficient to permit insemination at a predetermined time (104).

The intravaginal route for administration of progesterone and synthetic analogs (11) continues to be investigated, particularly in Ireland (30), France (73), New Zealand (91), and Israel (6). In heifers, at least, the potential value of the intravaginal approach has been underestimated (6). The New Zealand tests, involving 802 cattle, dairy and beef, suckling and dry, in which progestagen-impregnated intravaginal sponges were compared with PGF<sub>2α</sub>, led Smith (91) to conclude that, overall, the intravaginal sponge technique was satisfactory and offered greater potential for widespread use than did the prostaglandins. Loss of sponges by parous cows can be a problem, but it is dose-dependent. French workers have shown that, as in the sheep, the use of PMSG in association with the progestagen treatment (73) results in a high degree of precision of synchronization, 95% of cows treated in the first half of a normal cycle being in estrus within 72 hours.

A recent innovation is the development of a progesterone-releasing intravaginal device (PRID) consisting of a metal spiral coated with progesterone-impregnated silicone rubber. Following insertion into the vagina, the progesterone level in peripheral plasma rises to 5 to 10 ng/ml within an hour and remains high for 3 to 5 days. By 21 days it has declined to

TABLE VIII

**Treatment Parameters which Affect Fertility of Suckling Cows Treated with SC-21009 at Three Dose Levels for Three Periods, with or without Estradiol Valerate or PMSG<sup>a</sup>**

1. Duration of treatment (days)	9	11	13-15
Calving rate (%)	57.5	45.6	26.0
2. Estradiol valerate	5 mg	0 mg	
Calving rate (%)	52.8	19.7	
3. Dose of SC-21009 (mg)	12	9	6
Calving rate (%)	60.3	55.5	43.4
4. PMSG (IU)	800	0	
Pregnant at 21 days (%)	57.1	12.0	

<sup>a</sup> Optimum combination: 9 days treatment with 12 mg SC-21009 + 5 mg estradiol valerate on day 1 + 800 IU PMSG on day 10. Calving rate for optimum combination, 64.7% for first insemination and 82.7% for first 30 days.

<sup>b</sup> From reference (13).

2 ng/ml. On withdrawal, 100  $\mu$ g GnRH is given, and animals are inseminated 16–18 hours later. Good conception rates are reported (65).

## 2. Prostaglandins

Since 1972, when the first reports appeared of the intrauterine use of PGF<sub>2 $\alpha$</sub>  for inducing luteolysis in cattle (39, 57, 59, 84) and with the subsequent appearance and testing of systemically active salts and analogs, many reports have appeared evaluating the prostaglandins for synchronization. Two injections of 30 mg of the trimethamine salt (PGF<sub>2 $\alpha$</sub>  Tham; Upjohn) or 500  $\mu$ g of a synthetic analog (ICI 80,996) spaced 10–12 days apart, are reported to result in a high proportion of cows in heat between 48 and 72 hours after the second injection, with fertility to subsequent artificial insemination indistinguishable from normal (15, 54, 56).

## B. ARTIFICIAL TWINNING

There have been three major projects aimed at increasing the twinning rate in cattle.

The British trials (31), in which multiple ovulation was induced by PMSG given on day 16 or 17 of a normal cycle, resulted in a high proportion of triplets, quadruplets, and quintuplets. The response to PMSG was unpredictable and variable.

The French program (73), based upon the use of PMSG and HCG, concentrated upon (a) the responses obtained with these hormones when injected at various doses and times, (b) the variability of response depending on season, breed, and nutrition, and (c) fertilizability and survivability of superovulated oocytes. The work showed marked differences between breeds in the ovulation response and a curious phenomenon of "split estrus" in which there were two peaks of estrous response, some 2½ days apart. Despite a general trend for the mean number of ovulations to increase with increasing dose of PMSG, there was no significant linear response and the variability was enormous. However, within breeds, it was possible to class the animals into populations which would yield 0–1, 2–4, and >4 ovulations following a given dose of PMSG. There were breed differences, the Charolais proving more responsive than the Friesian.

The program in Ireland is in its infancy but a progress report is available (29).

## C. EMBRYO TRANSFER AND FROZEN STORAGE

Progress in these important areas of control of reproductive phenomena is discussed in Chapter 11.

## D DIAGNOSIS OF PREGNANCY

The assay of progesterone level in plasma (75, 80, 96) or milk (26, 45) on the 20th day after insemination promises to be a reliable test for early pregnancy, and is a potentially useful tool for studying embryonic mortality (76). A level of  $<0.5$  ng/ml plasma or 2 ng/ml milk is classed as nonpregnant and  $>2$  ng/ml plasma or 11 ng/ml milk as pregnant. False negatives are virtually nil while false positives can be attributed to embryonic mortality. There are practical problems associated with the collection and assay of blood plasma (96) which makes the milk test the more attractive.

## E INITIATION OF PARTURITION

The use of exogenous estrogen, prostaglandin, or corticosteroid for the induction of parturition is considered in Chapter 13.

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# 17

## Reproduction in Pigs

Philip J. Dziuk

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## I. Introduction

Pigs are litter-bearing mammals that can conceive and bear young at all seasons of the year. Because they have been domesticated for several centuries farmers have known for most of this period that the interval between heats is about 21 days and that the gestation period is about 114 days.

Pigs have several characteristics that, when combined, make them somewhat unique among farm and research animals. They are curious, intelligent, easily trained, readily available in large numbers, and can be kept in confined areas quite easily. In recent years, experimental research has given us additional insight into the normal functioning of the reproductive processes, especially in the sow. This chapter will deal primarily with this information.

## II. Puberty

### A. GILTS

First estrus may occur when the gilt is 4-9 months of age. The plane of nutrition may slightly influence the age at first heat but much of the variability of age at puberty seems to be associated with the genetic background of the gilt and the physical and perhaps social environment (9, 17). Gilts kept in large groups or in relatively close confinement or isolated from boars have delayed puberty. Movement of gilts from one environment to another when they are about 6 months of age will often trigger the first heat within 5 to 6 days (46). The injection of a gonadotropin such as pregnant mares serum gonadotropin (PMSG) into prepubertal gilts older than 4 months will induce estrus and ovulation (2). Thus the ovaries are functional but are lacking sufficient stimulus prior to spontaneous first heat.

When the first estrus and ovulation occur spontaneously, mating, conception, pregnancy, and parturition proceed with about 70% of gilts farrowing. When ovulation is induced at 4 to 5 months of age, not all gilts show heat and the proportion of gilts conceiving and implanting embryos is reduced with only an occasional gilt producing a litter. As the age of the gilts at induction of ovulation increases, so does the fertility by such measures as proportion showing heat, conception rate and litter size. Puberty can be considered to occur only when all the components of reproduction are established. Much is still unknown about the influence that diet, social environment, number and density of animals per pen, and light and temperature have on onset of puberty in gilts.

## B. BOARS

At a few weeks of age, boars will show initial sexual interest but are not fertile until at least 4 months of age when sperm appear in the ejaculate. Young boars will mount pen mates at an age when the prepuce and glans penis are still not separated. Under the continual influence of androgens the various components of male sex behavior develop with the production of seminal fluids and sperm. Both prepubertal and pubertal experience with gilts and boars are essential to development of normal sex patterns.

## III. Estrous Cycle

### A. LENGTH OF CYCLE

The expected length of the estrous cycle is 21 days, with 18 to 24 days considered within the normal range. The day of heat is usually counted as day 0 followed by day 1. When a large number of estrous cycles are recorded in mated gilts there are two peaks in the frequency of intervals between heats. One occurs near 21 days and another is at 26 days. This 26-day interval is also found in mated gilts with only one to four fertilized eggs (38). Presumably a small number of embryos prolongs the normal 21-day interval but does not initiate a full-term gestation.

Estrous cycles occur throughout the year with no obvious seasonality. Lactation will inhibit estrus for 30 days or more. Estrus will ordinarily occur at 3 to 7 days after the litter is weaned if lactation has persisted for 15 days or more. Reduction in the number of pigs per litter or increasing the interval between nursings has been used as a managemental tool to attempt to induce estrus during lactation. Response to such treatments is variable but not reliable at this time. Estrus can be readily induced with PMSG any time after the 42nd day of lactation, but it is necessary to use great caution to observe signs of heat (8a).

Severe nutritional, disease, or social stress is often associated with anestrus. Improvement in conditions or change eliminating the stress are accompanied by return of estrous cycles.

### B. SIGNS OF ESTRUS

Estrus is characterized by a series of gradual behavioral and physiological signs (46). In the young gilt, in particular, the vulva swells and develops a reddened appearance often several days before other signs appear. This reddening and swelling is apparently due to the secretion of estrogen by developing follicles because administration of estrogen will also cause vulvar swelling and reddening. Following this swelling of the

vulva, the gilt may be very alert to the presence of the herdsman or the boar and be quite restless. She may possibly adopt a malelike sexual behavior as shown by her pursuit of other females and mounting and nuzzling of the flanks. She may show a great interest in a boar and tease him but will assume an aggressive stance and playfully fight him rather than be receptive to his attempts at mounting. At 24 hours before estrus the uterus is very tightly contracted and an examination of the internal reproductive organs by laparotomy will reveal follicles on the ovaries ranging from 8 to 12 mm in diameter. Cloudy mucus is sometimes seen at the opening of the vulva at this stage. Older multiparous sows are less likely to show vulvar swelling but will often display the same proestrous behavioral signs as gilts. During the early stages of receptivity, the gilts' activity and restlessness are accentuated with frequent seeking of attention by the herdsman or boar. They often emit a peculiar growling sound not unlike a boar. In response to the sound, smell, and nuzzling of the boar they assume a rigid, immobile receptive stance. This rigid stance may also be elicited by the herdsman in the receptive gilt by applying pressure to her back.

Success in artificial insemination of swine is dependent primarily on accurate detection of heat to ensure that inseminations are performed at the time relative to ovulation for optimum fertility. Supervised mating or artificial insemination breeding programs must be accompanied by a patient and persistent check of all animals at least twice daily. Details of the time for insemination will follow in Section V.

### C. HORMONAL LEVELS

The levels of hormones in the blood are changing continuously, which reflects the dynamic condition of the reproductive system during the cycle (23, 24). These changes are depicted in Fig. 1 showing fluctuations of LH, progesterone, and estrogen. The level of progesterone is correlated with the number of ovulations during the first 8 days but the relationship is much reduced after day 12.

The time of release of LH coincides with the onset of heat with little evidence for diurnal rhythm. The ascending slope of the peak of LH is relatively shallow and the peak is lower than in the sheep and cow (36). The levels of FSH vary little throughout the estrous cycle (41).

## IV. Ovulation

### A. TIME

Ovulation of follicles occurs near the end of the estrus on about day 2 of the cycle. The interval from injection of human chorionic gonadotropin

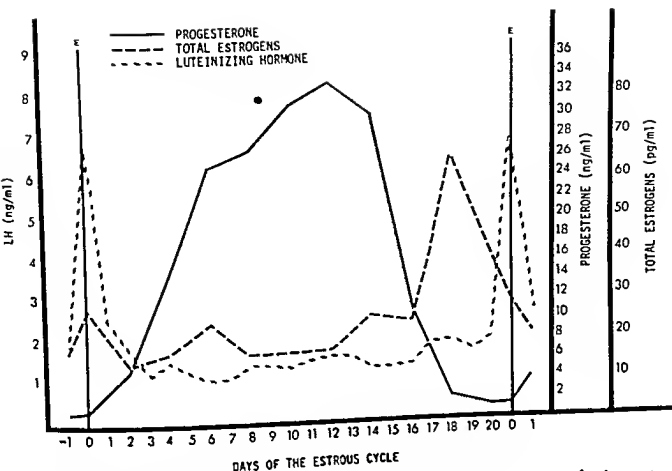


FIG. 1. Peripheral plasma levels of luteinizing hormone, progesterone, and estrogens during the porcine estrous cycle (Hansel *et al.*, 24).

(HCG) to ovulation is  $41 \pm 1$  hour in gilts in late proestrus or in gilts with mature follicles produced after withdrawal of ovulation-inhibiting materials (26, 27). In normal cyclic gilts the interval from release of endogenous pituitary LH and ovulation is also about 40 hours. Release of LH is at the onset of heat (36). The administration of GnRH will also cause release of LH and ovulation (3).

## B. NUMBERS

The number of eggs ovulated is influenced by many factors. The mean number of eggs ovulated increases from 8 to 10 at the first postpubertal heat to 12 to 14 at the third heat. Sows will ovulate 15–20 eggs (17).

If gilts are slightly undernourished, the number of ovulations is somewhat less than in gilts with a greater caloric intake. An increase in caloric intake by supplying additional feed at least 7 days before the next anticipated ovulation will result in an increase in two ovulations compared to females not given additional feed (19, 35).

Certain breeds of pigs ovulate more eggs than do others. Recent evi-

dence has shown that the number of ovulations is highly heritable indicating a genetic basis for ovulation rate.

### C. DEVELOPMENT OF FOLLICLES AND CORPORA LUTEA

The stage of the estrous cycle may be determined quite precisely during the first 8 days after ovulation and for the 5 days preceding ovulation by examining the ovaries. This leaves only days 8 through 15 with little easily distinguished daily change. Beginning at day 14 or 15 of the estrous cycle and progressing onward, the ovarian changes are as follows: corpora lutea begin to regress developing large surface capillaries and a generally paler appearance; at day 16-17, corpora lutea may be regressed to 4 to 5 mm in diameter and are now whitish, hard, and avascular and follicles are 4-5 mm in diameter; at day 18-19, corpora lutea are now 3 to 4 mm and are white (corpora albicantia), follicles are 10-12 mm in diameter, quite turgid, and clear walled with many fine capillaries; at day 20-21, follicles have lost their turgidity and are somewhat pendulous with very clear walls, ovulation is imminent and occurs about 24 hours later at which time the follicles have collapsed, the ovulation stigma is evident and bloody, with a small amount of blood oozing from it, and there is little luteal tissue; at 24-48 hours, the follicle is 10-12 mm, filled with a clear or bloody gelatinous material, still oozing blood, with a very slight pinkish tinge to the wall from luteal tissues; at 72 hours, follicles are 12-15 mm with pink color from luteal tissue lining follicle wall, and a small blood clot in center; at 96-120 hours, corpora lutea are 10-20 mm, very bright purplish with luteal tissue and a large blood clot is evident; at day 6-8, corpora lutea reducing to 10 to 12 mm with thick layer of luteal tissue and uniform pink color, small cavity is filled with fluid or blood; at day 9-14, corpora lutea pink, fine capillaries, solid structure with radial appearance when cut through a diameter; this state persists through pregnancy or until regression begins if pregnancy does not ensue.

## V. Fertilization and Embryonic Development

### A. SPERM PENETRATION

Insemination of gilts running with boars ordinarily takes place during estrus several hours before ovulation. The optimum time for insemination seems to be about 12 hours before ovulation (16). Sperm transport to the site of fertilization in the ampulla takes only a few minutes, thus, sperm

are ordinarily waiting for the egg. Attachment of sperm to the zona pellucida occurs within 1 or 2 hours after ovulation and sperm may activate a pig egg within 2 hours after insemination (26-28). Whereas the pig egg is ovulated in a cumulus mass, sperm rarely are found in the cumulus and attachment of sperm to the zona pellucida occurs after the egg is nearly completely denuded. Sperm penetrate the zona pellucida following a parabolic path beginning with a shallow angle and continuing with an ever increasing angle to the surface of the egg. Normally only one sperm penetrates the zona pellucida completely. The zona blocking reaction permits many accessory sperm to only partially penetrate the zona pellucida where they are trapped. Delayed mating or other conditions which permit large numbers of sperm in the vicinity of the recently ovulated egg is associated with polyspermy. The egg can distinguish between the first penetrating sperm and subsequent sperm only if some time elapses between them (29).

## B. DEVELOPMENT

The location and stage of development of pig embryos can be predicted quite accurately when the time of ovulation is known or, conversely, the time of mating can be estimated by examining recovered embryos (27).

After activation of the egg by the sperm, pronuclei form and fuse. Pronuclei can be found from 6 to 18 hours after ovulation. The egg undergoes the mitotic division to two cells at about 20 hours after ovulation. The embryo is four cells at about 30 hours and remains at this stage until 48 to 56 hours when it enters the uterus. At 72 to 96 hours, the embryos are at the morula stage, containing six to thirty-two cells. At day 6, nearly perfectly spherical blastocysts are formed with a distinct inner cell mass at one pole. At day 8 to 9 the blastocyst may collapse and be slightly elliptical. Blastocysts continue to elongate to day 10, ranging in size from 5 to 10 mm in diameter, while resembling a very white, collapsed hollow ball (13). Blastocysts are 20-300 mm in length and are readily visible to the naked eye by day 11. Trophoblast tissue is growing very rapidly and by day 12 to 13 the trophoblast is several centimeters in length and is similar in appearance to a long white, fragile bit of mucus. The length and amount of tissue increases to day 17 when blood and a heart beat appear in the 12- to 15-mm embryo. The uterus may show a barely perceptible swelling at day 21 at the site of the 18-mm embryo and fetal fluids. By day 25, fetal shape is apparent in the 25-mm pig. The head, limbs, and internal organs are evident. The amniotic cavity is well developed and the uterus is now noticeably enlarged. This fluid is detectable by ultrasonic echo analysis and is useful as a means of pregnancy diagnosis (31).

## VI. Embryonal Spacing

### A. TIME OF MIGRATION

Embryos enter the uterus about day 4 of the cycle, 48 hours after ovulation. They remain in the tip of the uterine horn during days 5 and 6. At day 7, embryos can be found at some distance from the uterotubal junction but even at day 8 embryos have not migrated into the opposite uterine horn (13). During days 9, 10, 11, and 12, the embryos occupy both horns (Figs. 2-4). Migration stops at this time (37).

### B. SPACING

During migration embryos from one horn enter the opposite horn in nearly every case. Embryos from each horn are mixed with embryos

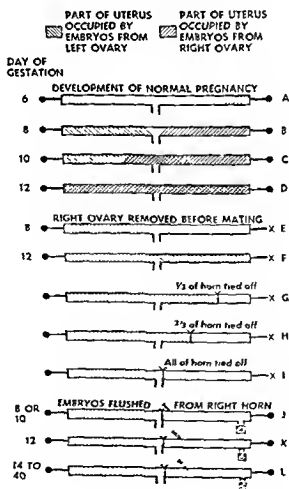


FIG. 2. Migration of pig embryos in early gestation (Dziuk, 14a).



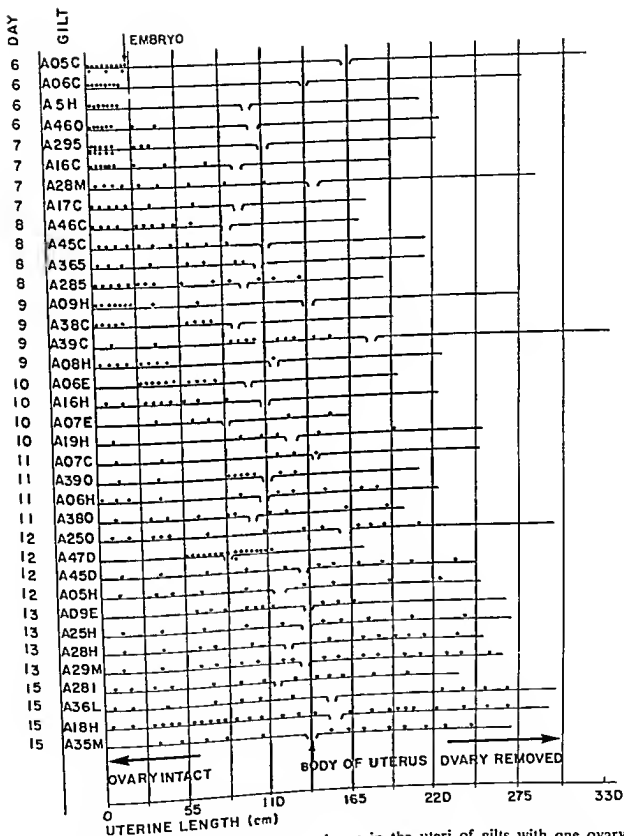


FIG. 3. Number and distribution of embryos in the uteri of gilts with one ovary (Dhindsa *et al.*, 13).

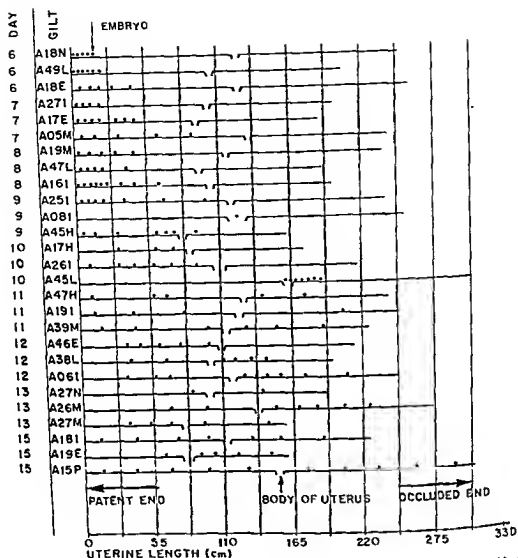


FIG. 4. Number and distribution of embryos in the uteri of gilts with one oviduct occluded (Dhindsa *et al.*, 13).

originating in the other horn. When one considers that perhaps ten or more embryos must share the uterine space which may range from 160 to 330 cm in length, for the 112- to 114-day gestation period, it is imperative that an efficient distribution mechanism ensure full utilization of available space. Embryos must be spaced approximately equidistant from each other.

When embryos are restricted to a shorter than usual segment by ligation of the uterus, they implant closer to each other than normal but still are equidistant from each other (15).

## VII. Pregnancy

### A. POSITION OF EMBRYOS

Not only is embryonal spacing and migration important to fully utilize uterine space, but it is essential for pregnancy maintenance (1, 22). When embryos occupy only one uterine horn or half of each uterine horn at day 12 to 14 of gestation the pregnancy will not continue and the sow will return to heat (11, 12). When one-third of the uterus is unoccupied, pregnancy can continue but the proportion of gilts maintaining pregnancy is less than in gilts with a completely occupied uterus. When fetuses occupy only one uterine horn at day 10, pregnancy does not continue but when fetuses are removed from one horn at day 12 or 14, a unilateral pregnancy can continue. When all fetuses are killed at 30 days of gestation, the corpora lutea continue functioning, the progesterone in the blood remains at the same level as during normal pregnancy, and the sow does not return to heat for at least 60 days (51). It appears that the sow must have at least 70% of the uterus occupied by embryos between day 10 and 12. Once the pregnancy has been established at day 14 or later, removal of part of the litter or perhaps death of all embryos does not disrupt the course of pregnancy.

### B. NUMBER OF EMBRYOS

An apparent safeguard against a sow maintaining a pregnancy initiated by less than four embryos at day 12 is evident from observations on limiting the number of embryos (38). When only one or two embryos entered the uterus, the estrous cycles were extended to 25 to 30 days. Pregnancy was not maintained to 40 days unless at least four embryos were present.

These observations, plus those mentioned above help us understand how the number and position of embryos influence whether the mated sow will return to heat or maintain the pregnancy. Once pregnancy has been established just before day 14, then it will continue with few or essentially no fetuses.

### C. MECHANISM OF SIGNAL FOR PREGNANCY

It is a truism that the difference between a nonpregnant sow and a pregnant one is the presence of embryos. The mechanism by which the 12-day embryo converts the normal cyclicity to a state of abeyance for an additional 100-day gestation is still an enigma. Hysterectomy or administration of an estrogen during the luteal phase will prolong the life of the

us luteum for a period nearly equal to gestation (22). The presence of embryos for 14 to 30 days will also often delay the next heat for several months. Unilateral regression of corpora lutea on the ovary adjacent to an unoccupied uterine horn while corpora lutea are maintained on the other ovary adjacent to an occupied horn indicates the possibility of a local effect of the uterus on the ovary. However, when ovaries are transplanted surgically to the uterus or to the abdominal body wall between muscle and the skin, estrous cycles occur normally (32, 50). When embryos are transferred into the uterus of gilts with transplanted ovaries, pregnancy ensues and parturition and lactation are normal. These transplanted, isolated ovaries can apparently respond to a systemic signal and need not rely on possible local routes. Unilateral pregnancies can be maintained by administration of progesterone or by developing an accessory corpus of corpora lutea during days 14–21 after mating (7). The exact mechanisms as to how the pig embryos signal the mother to maintain pregnancy is still not clear.

#### 4. HORMONAL LEVELS

The pig, unlike the sheep and human, requires the ovaries and corpora lutea throughout gestation. It may be assumed, therefore, that the contribution of the ovaries is essential and whatever contribution the fetus or placenta make are not sufficient to maintain pregnancy. The level of progesterone during the first 14 days of gestation is the same as the first 14 days of the estrous cycle (51). The level of progesterone in plasma from day 14 onward in pregnancy is maintained and does not fall to zero as in the cyclic gilt. Plasma progesterone gradually declines from the high of 20 to 40 ng/ml at day 14 to 6 to 10 ng/ml at day 110 (23, 25). There seems to be little effect of the number of fetuses on the level of progesterone. The level of progesterone is not correlated to the number of embryos surviving (34). Plasma progesterone levels remain within the normal range and corpora lutea remain functional even after all fetuses are dead (50). The observation that sows can be detected as pregnant at 40 days but fail to farrow can be explained on the basis that fetuses died after day 40 but the corpora lutea continued to function for an additional 60–90 days. Estrogen levels rise at day 23–30 decline and then rise again at parturition (43).

The minimum level of progesterone for maintenance of pregnancy is near 6 ng/ml in plasma (20). At lower levels the pregnancy is lost and higher levels do not appear to increase embryonal survival. It seems that the level of progesterone needed to preserve the pregnancy is an all-or-none phenomenon with little quantitative effect from various levels.

Prepubertal gilts at 4 to 5 months of age can be induced to ovulate and conceive but few carry pregnancy beyond 25 days. The uterus apparently can accommodate the fetuses but the corpora lutea do not persist, and the pregnancy is not maintained. Administration of exogenous progestins by the oral or parenteral route will maintain the pregnancy (21). Attempts to maintain the corpora lutea of pregnancy by administration of gonadotropins or by other presumed luteotropins have met with varied success. This indicates that the prepubertal gilt does not or cannot respond to the signals from her embryos as does the sexually mature gilt.

### VIII. Embryonal and Fetal Survival

Litter size at birth is affected by the number of eggs ovulated, the proportion of eggs fertilized, and finally the proportion of embryos and fetuses surviving to term. The number of eggs ovulated has been considered earlier in Section IV.

About 28 to 30% of all embryos die and are resorbed by day 30 of gestation. Ordinarily only part of each litter in each sow is lost, thus it is apparently not a contagious disease which spreads from sow to sow or embryo to embryo (44). There are some contagious diseases that do cause abortion or resorption but these account for relatively few losses and are often associated with abortion and mummified fetuses. Nutritional and managerial regimens have been found to have little consistent effect on the proportion of embryos surviving (6). A small proportion of sows do carry all embryos to term with no loss. Therefore, the loss is not innate.

#### A. SPACE

There are a number of possible explanations for embryonal losses. One possibility that has been explored quite thoroughly is that each embryo has a minimum requirement for intrauterine space (15, 40). When less than the minimum space is available, some embryos die because of the finite size of the uterus, the large number of embryos, and the length of the embryonic membranes. Several studies have found that the uterus can accommodate about twice as many fetuses up to day 30 than are normally present (8, 10, 15, 39, 40). Space is not limiting at 30 days but at 40 days space does limit the number of viable fetuses (51) (Fig. 5). Each uterine horn can accommodate about six or seven fetuses to advanced gestation and possibly to term. One might surmise from this observation that the mean litter size for a normal sow with both uterine horns

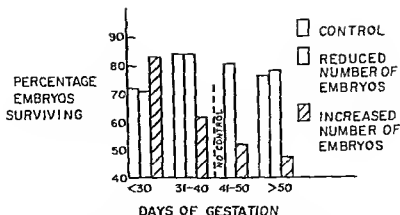


FIG. 5. Effect of altering the proportion of uterine space available to embryos on their survival during early gestation (Webel *et al.*, 51).

would be two times six or seven for a total of twelve to fourteen fetuses. Many sows do realize the full potential while others fall short.

## B. HORMONES

Because progesterone is one of the hormones essential for maintenance of pregnancy it would seem reasonable to think that slight deficiencies in progesterone levels in pregnant sows may be associated with a reduction in the proportion of embryos surviving. Several experimental attempts to supplement the normal levels by administration of exogenous progesterone have yielded equivocal results. Evidence obtained by experimentally reducing or increasing the number of fetuses and corpora lutea has shown that the number of fetuses does not influence the level of progesterone in the blood (51). The level of progesterone bears little relationship to the number or proportion of fetuses surviving providing the concentration of progesterone in blood plasma does not fall below about 6 ng/ml when the entire litter is lost (20).

## C. BOAR EFFECT

Evidence obtained from careful records of the litter size of many sows mated or inseminated to specific boars has shown that the boar can have a marked effect on litter size. Because the boar or his sperm are not likely to have an effect on the number of ovulations and because under usual circumstances nearly 100% of eggs are fertilized this would indicate that embryos and fetuses sired by certain boars are less likely to survive to term than those sired by other boars. This, in turn, leads one to think that genetic or fertilization errors may make a major contribution to embryonal

loss. Additional work will be needed to determine the extent of the influence of the boar on embryonal survival.

## IX. Parturition

### A. LENGTH OF GESTATION

"Three months, three weeks, and three days" is an easy way to remember the gestation period of pigs. The normal range can be from 109 to 120 days with most near 114 days and very few at the extremes. The length of gestation may be influenced genetically as there is some evidence that breed of sow and breed of boar may be involved. The number of fetuses in the litter can have an effect with smaller litters being carried slightly longer than large litters. The fetus and specifically the fetal head and presumably the fetal pituitary have a primary influence on gestation length (5, 49). Fetuses decapitated in the uterus at day 45 and allowed to grow and develop will rarely be born before day 125. Potent adrenal corticoids, given at high doses, will shorten gestation. Inhibition of the maternal pituitary has little effect on gestation length.

There seems to be little local effect of the fetus or uterus on the ovary in initiating parturition. Sows with ovaries separated from the uterus and transplanted to the body wall can be made pregnant by transfer of embryos. Such sows have gestation lengths that fall well within the usual range (32).

### B. HEAD-TAIL ORIENTATION

Slightly more than one-half of pig fetuses are presented head first at birth. This is much less than the 95% in cows, sheep, and humans (42). Some observers have found that the proportion of fetuses presented head first is greatest in the first one-half of the litter whereas others found fetuses were presented head first about 50% of the time at the beginning of farrowing with the proportion increasing to 70% in the last one-fourth of the litter (Fig. 6). This last observation has been corroborated by noting the head-tail orientation of fetuses in the uterus. At the tip of each horn the heads of fetuses were toward the cervix about 64% of the time regardless of whether they had originated from the ovary adjacent to that horn or had migrated throughout the entire length of the uterus. The means by which the embryo, at an early stage of development, is oriented with its head toward the cervix in the convoluted uterus is a puzzle.

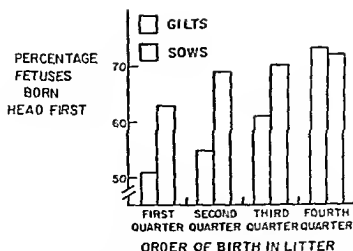


FIG. 6. The relationship between anteroposterior presentation of fetuses and their order of birth (Reimers *et al.*, 42).

### C. ORDER OF BIRTH

When the position of each fetus in the uterus is recorded, and each fetus is designated individually by a distinguishing mark during late gestation, and the order of birth is noted subsequently, several conclusions can be reached (Table I). Fetuses do not pass each other in the uterine horns at parturition; those nearest the cervix are born before those toward the oviduct (18). Ordinarily fetuses are presented from each uterine horn more or less at random so that one uterine horn does not empty completely be-

TABLE I  
Uterine Position of Fetuses and Order of Birth in the Sow

Sow identification	Order of birth of fetuses*		
	Left horn	Body	Right horn
A	9-8-5-2		1-3-4-6-7-10
B	6-5-2-1		3-4-7-8-9-10
C	7-6-5-3-1		2-4-8-9-10
D	13-11-10-9-8-5-2-1		3-4-6-7-12-14
E	10-8-6-4-3-2		1-5-7-9-11
F	9-6-5-2-1		3-4-7-8-10
G	6-4-3-2		1-5-7-8
H	9-5-3-2-1		4-6-7-8-10-11-12
I	11-10-8-7-6-4-2		1-3-5-9-12
J	11-10-9-8-2-1		3-4-5-6-7
K	10-9-8-7-6		1-2-3-4-5

\* The numbers indicate the order of birth of fetuses.



fore some fetuses come from the other horn. Head-tail orientation remains essentially unchanged during pregnancy and parturition with relatively few fetuses reversing their head-tail orientation.

#### D. TIME INTERVAL BETWEEN PIGLETS AND STILLBIRTHS

About 5 to 10% of fully formed normal piglets alive before the birth process begins are farrowed dead or are stillborn (47, 48). Because their lungs have not inflated they are presumed to die during the birth process. The mean interval between birth of a piglet and a subsequent live litter mate has ranged from 13 to 18 minutes. The mean interval from a piglet to a stillborn is 45-55 minutes. A delay in passing from the normal location in the uterus to the outside is associated with a high incidence of stillbirths. The incidence of stillbirths is much higher in litters of less than four piglets or greater than nine piglets. The incidence of stillbirths is also greater in the last few piglets born from the tip of the uterus than in other piglets (Fig. 7). About 70% of stillborn piglets are in the last three pigs born when the interval between piglets increases (Fig. 8).

#### E. INITIATION OF PARTURITION

As mentioned previously in Section IX,A, the fetal head and presumably the pituitary play an important role in initiating parturition. The fetal adrenal in decapitated fetuses is very small, possibly due to lack of pi-

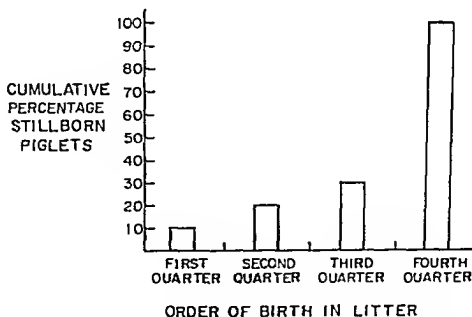


Fig. 7. The relationship between incidence of stillbirths and order of birth (Dziuk *et al.*, 1960).

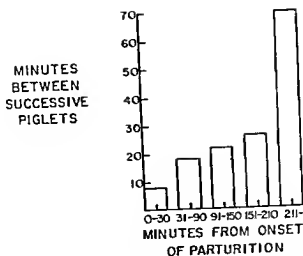


FIG. 8. The relationship between time from onset of parturition and the interval between births (Dziuk *et al.*, 18a).

tuitary hormone stimulation. This lack of adrenal function may be responsible for the prolonged gestation in litters of decapitated fetuses. Further support for fetal adrenal involvement comes from previously mentioned research in which a potent synthetic adrenal cortical steroid shortened the gestation period. Smaller doses given to fetuses in the uterus also induced parturition indicating the possibility that the fetal adrenal is responsible.

Removal of the ovaries or the corpora lutea at all stages of gestation results in birth of fetuses in 30 to 48 hours. Administration of potent luteolytic substances, such as prostaglandin  $F_{2\alpha}$ , during pregnancy will cause birth of fetuses in about 30 hours in most pregnant sows (14). Normally, at about 24 hours before parturition, levels of progesterone in the blood drop to less than 2 ng/ml coinciding with the regression of the corpora lutea. At this time there is no definitive evidence that prostaglandins are the cause of the normal regression of the corpora lutea just prior to a parturition. When a second set of corpora lutea are induced at day 109, gestation is prolonged to about day 120 indicating the intrinsic 14-day life of pig corpora lutea and the need for corpora luteal regression before parturition can take place.

#### F. LEVELS OF HORMONES

The concentration of progesterone and estrogens in the blood during the last few days of gestation and shortly after parturition changes constantly and quite sharply (4, 30, 33). Levels of both hormones have remained relatively constant throughout gestation with progesterone gradually subsiding and estrogens rising. Corticoids, which have been present at a low but constant level, now suddenly reach a peak many times the usual level.

Relaxin is found in high concentration in corpora lutea of swine a few weeks before parturition but blood levels are low, a few days before parturition the level in the blood rises very rapidly reaching levels as much as 50 times the level at day 105 (45). At 24 hours after parturition, both the contents in the ovary and in the blood drop to barely detectable levels. To what extent the rise in relaxin levels in the blood induces parturition or to what extent it is a result of other factors acting at parturition is not known. A summary of the enigma of the endocrinology of parturition might be as follows. Progesterone gradually declines then falls sharply just at parturition, estrogen rises over a few days, reaches a peak about 16 to 24 hours before parturition, and falls, corticoids and relaxin follow the same pattern and at essentially the same time scale, cause and effect relationships between any of these hormones has not as yet been clearly established.

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# 18 Reproduction in the Ewe and the Goat

Hamish A Robertson

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## PART I THE EWE

In the past 15 years the ewe has become one of the preferred animals for research on mammalian reproduction. This has been a result of economic pressures to make livestock production more efficient and the realization that not only do sheep make useful experimental animals but also that comparative studies can lead to a better understanding of reproductive

processes, including those in man. Many of the major studies recently carried out on sheep have been done within the aegis of human medicine.

For a comprehensive appreciation of the breeding patterns in sheep it is necessary to recognize that current sheep production practices are divided into two systems. The classical extensive system is still the most widespread. It arose as a consequence of the relegation of sheep production to the poorer grazings. In this system, the time of the birth and the rearing of the offspring are closely related to climatic and nutritional conditions. The ewes are usually bred once a year during late autumn and the birth of the lambs coincides with the first flush of spring pasture, which provides sustenance first for the heavy nutritional demands of the lactating ewe and then for the growth of the weanling lamb. Except for the last 3-4 weeks of pregnancy, the growth of the fetus requires little increase in nutritional intake over the normal maintenance requirement. Under this system there has been no requirement and hence no selection for a prolonged breeding season. Because this system is found mainly in geographical locations outside the tropics, the breeding season of these breeds of sheep is still predominantly regulated by seasonal changes in day length. In addition, under conditions of suboptimal nutrition, as frequently happens on these poorer grazings, fecundity is depressed by a direct reduction in the ovulation rate or by a natural selection operating against prolificacy through the inability of the ewe to provide milk for more than one lamb. However, although prolificacy is naturally suppressed by nutritional status in some breeds it has not been lost. As an example, the ovulation rate of the Scottish Black Face ewe can be varied between a mean of one and two by changing the dietary intake (28). Breeds of sheep, such as this one, evolved in an extensive system of sheep management have, therefore, a restricted breeding season and normally give birth to only one lamb.

As social pressures in some countries tend to drive shepherds and, hence, sheep from the remoter rougher grazings to the more populous, lush, and more expensive land, another system of sheep production is evolving. This system is enforced by economic pressures. Here the requirement is for an intensive system in which, in order to satisfy both the marketing demands and the inherent high capital investment, marketable lambs have to be produced in batches at frequent intervals throughout the year and as early as possible in the reproductive life of the ewe. To meet the demands of this system the producer has turned to the reproductive physiologist to produce short-term solutions to problems such as out of season breeding, until such time as breeds of sheep are evolved by selection and by cross breeding which, like the present day dairy cow, will breed at any time during the year.

To meet these challenges, we need to understand the basic features of

reproduction of sheep and the endogenous control mechanisms regulating the various reproductive processes. Our understanding of reproduction in sheep has been markedly advanced during the last 10 years by the remarkable advances in methodology for measuring the concentration of hormones in blood.

## I. Puberty

In animals such as the sheep in which reproductive activity is related to a seasonally changing environment such as photoperiodicity, the age at which puberty occurs, i.e., the time of the first ovulation, is markedly affected by the time of year of birth. Lambs of early maturing breeds born in early spring (March–April) will cycle and conceive at 6 to 8 months of age, whereas those born in late spring (May–June) may not cycle until they are about 16 months old, i.e., the following autumn. The age to puberty and to first conception is economically important in an intensive sheep operation, but on the more marginal grazings it is not considered desirable to breed a ewe lamb until she is 18 months old.

## II. Seasonality of Sexual Activity

Over the centuries during which sheep have been domesticated, distinct breeds have evolved to conform to particular environmental characteristics and management practices. It is now possible to find a complete gradation in the duration of the annual season of sexual activity of the ewe, from the monoestrous condition of some wild species (38), through the seasonal polyestrous state of the majority of domesticated breeds, to the example of breeds such as the Merino which, in tropical environments, are able to reproduce at almost any time of the year.

In any breed of sheep, the duration of the breeding season may be considerably modified by the particular strain being studied, by its geographical location, climatic environment, and nutritional state. Therefore, the parameters quoted for the seasonality and the duration of sexual activity, are average values from which deviations, usually minor, can be found.

In the past the conventional method of determining the onset and termination of the sexual season of the ewe was by means of a marker ram. This method is dependent on the seasonality of libido of the ram. Such a method records behavioral estrus and not ovulation and it should be noted that the presence of a ram may, under certain conditions, stimulate sexual activity in the ewe outside the normal breeding season. The onset and termination

of ovulation can be determined by following the appearance and disappearance of corpora lutea. This can be done under laboratory conditions by repeated laparoscopy or by determining plasma progesterone levels in blood samples collected once or twice weekly. Ovulation will have occurred when the concentration of plasma progesterone rises above 0.8 ng/ml.

The ovulatory season of the ewe occurs during autumn and early winter; in northern latitudes the season extends from September to February for most of the lowland mutton breeds. Hill breeds have a more restricted season, whereas that of the fine-wool breeds, which have been developed in warm climates not subject to extreme seasonal climatic changes, is more prolonged. It is possible to recognize a variant of this seasonality of sexual activity. Some breeds, such as the Ile-de-France (74), may emulate the Barbary ewe which can exhibit two seasons of ovarian activity: one from October to January and the other from April to June. In this situation in which sexual behavior during the second period of ovarian activity may be absent, the introduction of an active ram may lead to full sexual activity and successful conception. This situation may also exist in the Australian Merino.

Under conditions of natural lighting the seasonality of ovarian activity is related to the ratio of the hours of light to the hours of darkness. The sexual season begins for different breeds at some fixed time interval between 60 and 120 days after this ratio starts to fall, i.e., after June 21st in northern latitudes. This, however, is only a generalization, because under both natural (74) and artificial light cycles (41; Robertson, unpublished data.), breeds of sheep or individual ewes that start to cycle when the light to dark ratio is still increasing can be recognized. Also ewes born and reared under a 24 hour continuous light regime show, in the presence of a ram, fairly regular periods of sexual activity interspersed with periods of anestrus (Robertson, unpublished data.).

Although much information on the experimental manipulation of the sexual season by altering the light cycle has been reported, many of the experiments, and the interpretation of the data obtained, may be criticized because the duration of the experiments was too short and the ewes were not born and reared under the experimental light cycles but were already entrained to light of a different periodicity, e.g., natural daylight.

There is evidence to indicate that in some species the pineal gland through its secretion of melatonin is involved in mediating the effects of light on gonadal function. Pinealectomy of the ewe, however, does not affect its seasonal breeding pattern (64). The plasma concentration of prolactin is higher in both the ram and the ewe during anestrus as compared to the breeding season and this finding merits further investigation.

Since the ram shows seasonal changes in libido and to some extent



spermatogenesis, the possible confounding effect of this must be recognized in the design of experiments relating to the manipulation of the sexual season of the ewe in which the ram is used to monitor her sexual activity. Since the seasonal changes in the libido of the ram can be directly correlated with the plasma testosterone level, which is low at the time of the seasonal anestrus of ewes of the same breed and high during the ewe's sexual season, rams would probably be better experimental models than ewes for studying the effect of manipulating photoperiod. Plasma testosterone levels could then be used to monitor the sexual season. On the other hand, the episodic releases of LH from the pituitary producing fluctuating levels of testosterone throughout the day (36) poses problems of sampling.

The relationship between photoperiodicity and ovarian activity is more complex than many reports and reviews would lead one to believe. This complexity is increased when differing responses of different breeds are considered. No successful continuous system of breeding ewes on a production basis at 6- to 8-month intervals by the use of controlled lighting alone, has as yet, been reported.

Seasonal changes in temperature do not override seasonal changes in natural light photoperiodicity. Ewes maintained in a simulated natural lighting regime 6 months out of phase with the normal seasonal lighting and hence environmental temperature have a sexual season during the warm weather of late spring and early summer and a period of anestrus during the colder autumn and winter months (Robertson, unpublished data). During hot weather ( $>25^{\circ}\text{C}$ ), particularly when exposed to unshaded sunlight, rams exhibit reduced libido. Although ovulation may not be affected in the ewe during periods of continuously high temperature ( $>35^{\circ}\text{C}$ ), early embryonic loss can be expected. This effect on reproductive efficiency is not so great when high temperatures during the day are interspersed with cool nights.

### III. The Estrous Cycle

#### A. GENERAL CHARACTERISTICS

The term estrus will be used to denote the period of time during which the female of most mammals will accept mating by the male. The onset of estrus defines the time at which the female first permits mating. Under normal conditions, the onset of estrus in the ewe is precisely related in time to other physiological events. The day on which the onset of estrus occurs will be denoted day 0. Since the time of the onset of estrus can readily be determined by using a vasectomized ram fitted with some device for marking the ewe when he mounts her, it is a useful reference point

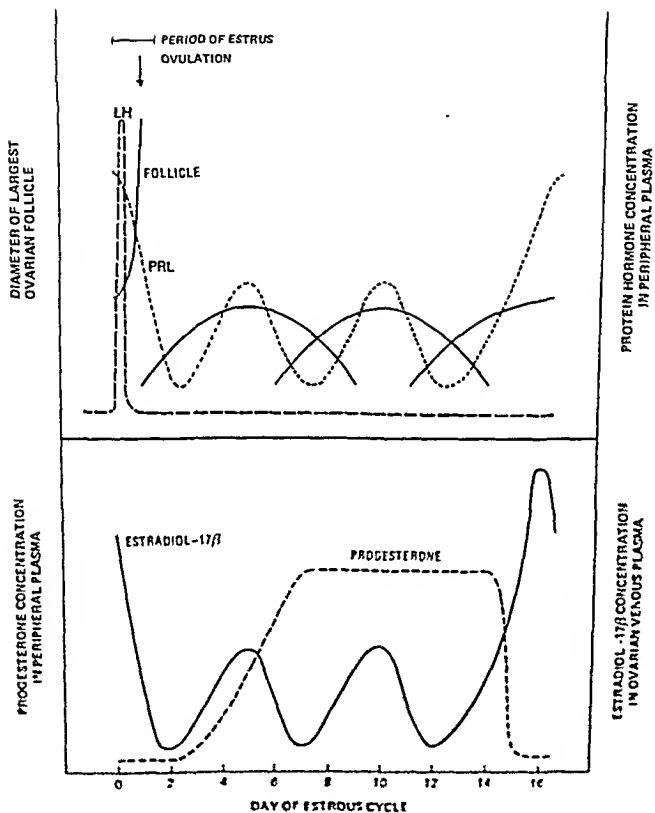
from which to time the various stages of the estrous cycle. It is largely because these subsequent events can be accurately timed that the ewe has become one of the favored experimental animals for research on reproduction.

The most common breeds of sheep have a mean interestrus interval of 16.5 or 17.5 days (2). The modal duration of sexual receptivity is generally about 30 hours, but the period may be longer for a few breeds such as the Merino and Finnish Landrace. Copulation at the time of onset of estrus may reduce the time during which the ewe is sexually receptive. Most reports concerning the duration of the estrous cycle are based on observations of evidence of mating made only once or twice a day. With this procedure, considerable errors in determining the duration may occur. Because it is much more difficult to determine when estrus ends than when it begins, accurate observations on the duration of estrus are limited.

The ewe is a spontaneous ovulator. In most breeds, ovulation, as determined by laparotomy, usually occurs 24–27 hours after the onset of estrus (26, 43), although it can occur later (54). Most breeds shed one or perhaps two ova. The Finnish Landrace and Romanov breeds regularly shed two to five ova. Experiments have demonstrated that the ovulation rate changes with the time of year and the age and nutritional status of the ewe. The ovulation rate is highest in the middle of the season of sexual activity, i.e., the optimum time for fecundity for ewes in northern latitudes is during November. Ewes attain their peak prolificacy at 4 to 6 years of age and are capable of carrying a lamb in their 18th year.

## B. THE GROWTH AND ATRESIA OF GRAAFIAN FOLLICLES

Immediately after ovulation has occurred no follicles larger than 2 mm in diameter can be found in the ovaries, but by day 3–4 a Graafian follicle, 4–5 mm in diameter, capable of being ovulated has appeared (Fig. 1). At least one such follicle can be found at any time of the cycle from day 3–4 until the next onset of estrus (32, 57, 67). It might be assumed that the 5-mm follicle observable early in the cycle is the one destined to rupture at the time of the next ovulation, but present evidence suggests that this is not so. A series of follicles grow and regress during the cycle, and the one which will ovulate is not discernible until 48–36 hours before ovulation, i.e., some 24–12 hours before the onset of estrus (73). If the ruptured follicle is destroyed by electrocoagulation just after ovulation so that a functional corpus luteum does not develop, the ewe will ovulate again about 4 to 5 days later. We might conclude from this evidence that the ewe has an intrinsic follicular and ovulatory cycle of 4 to 5 days in the absence of a corpus luteum and a 4–5 day follicular cycle without ovula-



tion in the presence of a corpus luteum. This situation is similar to that of the rat (73) and there are three or four discrete waves of Graafian follicular development followed by atresia during a normal estrous cycle. Ovulation of all but one of these follicles is suppressed by the presence of a functional corpus luteum. Some support for this concept is provided by the observation of a rise in estradiol-17 $\beta$  in ovarian venous blood on days 2, 8, and 14 (11, 50, 70) corresponding to maturation of successive follicles (Fig. 1).

The preovulatory enlargement of a Graafian follicle begins shortly after the onset of estrus (26) slowly at first, with growth becoming more dramatic at about +18 hours, i.e., 8–10 hours before ovulation (43). The maximum diameter of the follicle before ovulation is 1.2 cm (Fig. 1).

The growth and atresia of Graafian follicles presumably continues until some time about 60 days of gestation; 5-mm follicles can be identified at least up to this time. It is not clear whether the subsequent disappearance of 5-mm follicles is the result of a change in the endocrine control of follicular growth per se or whether it occurs as the natural consequence of a transition into the normal period of seasonal anestrus.

During seasonal anestrus, 5-mm follicles can occasionally be found in the ovaries. In the mature ewe, follicular, and hence ovarian quiescence, may be relative, with the breed of the ewe, the presence or absence of a ram, and the type of environment contributing to the degree of quiescence. Very little is known about the mechanisms controlling growth and atresia of Graafian follicles, a situation not unique to the ewe.

### C. SEXUAL RECEPTIVITY

Behavioral estrus in the ewe normally lasts 24–30 hours but this period varies. Estrus is induced by the effect of estrogen on the central nervous system. This can be shown experimentally by introducing implants containing very small quantities of estradiol-17 $\beta$  into the hypothalamus. Only very much larger amounts of estradiol-17 $\beta$  given systemically are effective in inducing behavioral estrus. Progesterone has been shown to have a synergistic effect with estradiol-17 $\beta$ . When it is present or when the hypothalamus has recently been exposed to progesterone, very low systemic concentrations of estradiol-17 $\beta$  (15 pg/ml) will induce sexual receptivity. The use of progesterone as a primer before treatment with pregnant mares serum gonadotropin (PMSG) to induce estrus accompanied by ovulation in anestrus ewes (15, 18, 62) led to the development of a system for "out-of-season" breeding of sheep.

During a sequence of estrous cycles, such as occur during the normal breeding season, it is fairly clear that a preconditioning period with pro-

gesterone precedes the rise in plasma estradiol-17 $\beta$  that induces behavioural estrus. However, what happens at the beginning of the sexual season? Ewes killed just before the first expected estrus of a new breeding season have corpora lutea in their ovaries (9, 25). This implies that in the ewe the first ovulation at the beginning of a new sexual season is not associated with behavioral estrus. The progesterone from the first corpus luteum of the season conditions the ewe so that full estrous behavior is manifested at the time of the estrogen peak preceding the second ovulation. Under normal conditions of husbandry, rams are segregated from the ewes. When the rams are introduced to ewes in the transitional stage between anestrus and the breeding season, the ewes ovulate within 6 days without exhibiting estrous behavior and show full estrous behavior 16–17 days later (22, 71, 76). Such an effect is also found when ewes can smell and hear the rams but have no visual or other contact with them (79). The behavioral aspects of estrus in sheep have been well-described (3).

#### D. OPTIMUM TIME FOR INSEMINATION

Rams are prodigious in their copulatory and ejaculatory capabilities. A ram can inseminate a single ewe many times within minutes. When there are a number of rams in the flock, a ewe may be inseminated by several rams while she is in estrus. The period during which one ram will "tend" a ewe in estrus varies. Although artificial insemination (AI) of sheep using freshly collected semen has been widely practiced in the USSR for many years, AI using fresh semen has been introduced only recently in other countries as an adjunct to synchronization of breeding. When freezing and long-term storage of ram semen become realities, AI of ewes will become more widespread. For AI to be successful, we must know the optimum time at which to carry out this procedure. A number of studies have shown that greatest success occurs when the ewe is inseminated about 10 hours after the onset of estrus with limits between 2 and 15 hours (19, 55, 72). It is of considerable interest that in wild or feral sheep, such as the Soay sheep on St. Kilda, Scotland, it is during the first hours of sexual receptivity that the dominant rams tenaciously "tend" ewes in estrus while subordinate rams spend more time and energy with ewes in late estrus, thus promoting selection of the dominant ram's genes (27).

#### E. THE CORPUS LUTEUM AND ITS MAINTENANCE

Ovulation and the initiation of luteal function may, at present, be regarded as synonymous events. They are preceded by a pituitary discharge of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), (57, 60, 67), and a corresponding rise in levels of plasma LH (13) and

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FSH (31). The release of both of these hormones may be attributed to the action of the estrus-inducing peak of estradiol-17 $\beta$ . Prolactin (PRL) likewise is secreted from the pituitary in large amounts at estrus (16, 34), also under the influence of estradiol-17 $\beta$ . Insofar as the initiation of luteal function is concerned, this preovulatory rise in plasma gonadotropins is associated with the entry of LH into the preovulatory follicle. At this time, the follicular fluid of the ewe contains >75 ng/ml of LH (5). It is probable that after ovulation, the LH receptor sites on the granulosa cells are occupied by LH and luteinization and secretion of progesterone are initiated when this LH stimulates adenylate cyclase.

Following ovulation the blood supply to the granulosa cells increases. These cells grow and divide forming a solid body, the corpus luteum, which attains its maximum morphological size (32, 56) and functional activity by about day 7 (73). At this stage, the corpus luteum is a highly vascularized gland, receiving approximately 97% of the total ovarian blood flow (49). This transformation or luteinization of the granulosa cells is accompanied by the synthesis and secretion of progesterone. From a basal concentration of 0.2 ng/ml, a discernible rise in the peripheral plasma concentration of progesterone occurs around day 4, reaching a plateau of 2 to 4 ng/ml by day 7, then rapidly declines on day 15, i.e., about 36 hours before the onset of the next period of sexual receptivity. The basal concentration of 0.2 ng/ml presumably arises from the adrenal cortex inasmuch as similar levels of progesterone are found in ovariectomized ewes and in intact ewes during seasonal anestrus.

The rise in the plasma progesterone which occurs by day 4 may suppress behavioral estrus and a discharge of pituitary gonadotropins, which would induce ovulation, in response to the pulse of estradiol-17 $\beta$  (11) secreted by the Graafian follicle present at this time (58, 73). It might be assumed that the ovulation of two ova, giving rise to two functional corpora lutea, would lead to twice the concentration of progesterone in peripheral plasma. Such, however, is not the case. Although there is evidence that the concentration of plasma progesterone in ewes with two corpora lutea is marginally greater than that of a ewe with one, it is not possible, unfortunately, to select individual ewes for twinning ability by measuring their peak plasma progesterone concentrations.

It may be argued that steroidogenesis by cells such as those of the granulosa layer require the support of gonadotropins and that, in the case of the nonpregnant, cycling ewe, this stimulus must come from the pituitary. Although the data are not entirely conclusive, evidence obtained with hypophysectomized ewes suggest that although LH and PRL individually are capable of sustaining a limited degree of progesterone synthesis, both are required for the corpus luteum of the hypophysectomized ewe to synthesize and secrete amounts of progesterone comparable to those found in intact



animals (17). Also, the infusion of antisera to LH or to PRL, leads to a reduction of progesterone secretion by the corpus luteum. Therefore, we must conclude that both LH and PRL contribute to the maintenance of the functional activity of the corpus luteum of the ewe during a normal cycle.

Figure 1 demonstrates that, as assessed by progesterone synthesis and secretion into peripheral circulation, the functional activity of the corpus luteum is terminated abruptly on day 15. Because there is no evidence for a withdrawal of luteotropic support by the pituitary, there being sustained moderate concentrations of LH and prolactin in blood at this time and, moreover, the decline in activity is too rapid to be due to withdrawal of pituitary support, some other active mechanism must be sought to account for the destruction or lysis of the corpus luteum. If ewes are hysterectomized during the active life of the corpus luteum, luteolysis does not occur, and its life span is prolonged for about 5 months, i.e., the duration of a normal pregnancy. Further experiments have shown evidence for the involvement of a local utero-ovarian interaction. (see Chapter 4). The concept of a local utero-ovarian cycle has been extended into the hypothesis that the functional activity of the corpus luteum is self-regulating: progesterone secreted by the corpus luteum stimulates the endometrium of the uterus to synthesize and store (80) the luteolytic substance. In the ewe this appears to be prostaglandin  $F_{2\alpha}$  ( $PGF_{2\alpha}$ ) (42). Increased levels of  $PGF_{2\alpha}$  have been found in the uterine venous blood of sheep on day 15–16 of a normal estrous cycle (6). As yet the mode of transfer of  $PGF_{2\alpha}$  from the uterine vein possibly into the ovarian artery has not been satisfactorily explained. Large doses of  $PGF_{2\alpha}$  given systemically, or smaller doses applied directly to the corpus luteum, stops the synthesis and secretion of progesterone within 6 hours. This can be used as an effective method of exogenously controlling the ovulatory cycle. It should be noted, however, by analogy with the cow that the corpus luteum of the ewe may not be luteolyzed by  $PGF_{2\alpha}$  during the first 4–5 days of its life span. The precise mode of its action in causing luteolysis is not known. As noted above, progesterone stimulates the synthesis and storage of  $PGF_{2\alpha}$  in the endometrium of the uterus but the rapid release that occurs on day 15 resulting in luteolysis remains to be explained.

Present evidence suggests that estrogen may be involved in the release of  $PGF_{2\alpha}$  from the uterus. Estradiol-17 $\beta$ , if given in a large enough dose, will induce luteolysis when given toward the end of the estrous cycle and therefore is a prime suspect as the stimulator of the release of  $PGF_{2\alpha}$  from the uterus. Support for this view comes from the finding that the output of estradiol-17 $\beta$  in ovarian venous plasma of the ewe rises 48 hours before the onset of estrus, i.e., just before the functional activity of the corpus luteum ceases (5, 48, 70).

Many experiments have shown that artificially prolonging the luteal phase of the cycle by the administration of a progestagen delays ovulation, but not luteolysis, and that ovulation occurs in the absence of a functional corpus luteum, only when the progestagen block is removed. Also, if the corpus luteum is enucleated during a normal cycle, ovulation occurs about 70 to 80 hours later. These experiments show that the duration of a normal estrous cycle is governed by the life span of the corpus luteum. But how does the lowering of the peripheral plasma level of progesterone initiate the next stages of the estrous cycle in the ewe, viz, behavioral estrus and ovulation?

## F. OVULATION

For some 26–48 hours before the onset of estrus, i.e., 72–60 hours before ovulation, a 4-mm follicle can be recognized as the one that will ovulate (73) by which time it has commenced to secrete estradiol-17 $\beta$  (with very little estrone) (5, 48, 70). By 30–24 hours before estrus it has attained a diameter of about 5 mm but at this time it does not differ morphologically from other 5-mm follicles that were present earlier in the cycle. The secretion of estrogen, as measured in ovarian venous blood, reaches a peak about 24 hours before the onset of behavioral estrus and drops again by +8 hours (Fig. 1), i.e., approximately 20 hours before the cessation of sexual receptivity (48). Somewhat surprisingly, the maximal level of estradiol-17 $\beta$  in peripheral blood at this time is only about 15 pg/ml, which is only about twice the basal level found during the period of high progesterone secretion. It is difficult to accept that there is only a twofold safety factor between the basal estrogen concentration and that required for the induction of behavioral estrus, although the physiological effects of estrogen are enhanced by the previous exposure to progesterone.

What initiates estrogen synthesis by the preovulatory follicle? It is first necessary to recall that the preovulatory follicle is not unique in its ability to secrete estrogen; other Graafian follicles, which mature in a regular sequence, also secrete estrogen at specific periods during the cycle. As yet, assay of the plasma levels of the two gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH), has failed to reveal any meaningful change in their concentration at the time estrogen secretion is initiated. It is possible that at this time it is not changes in the concentration of these pituitary gonadotropins in blood which are important but rather that the number of gonadotropin receptor sites on the follicle is modified by other hormonal changes with the result that the sensitivity of the follicle to a fixed level of gonadotropin changes. Whatever the mechanism, estrogen is synthesized by the theca interna cells. As noted above, synthesis and secre-

tion of estrogen by the preovulatory follicle is suddenly shut off at about +8 hours after the onset of behavioral estrus, and there is some evidence to indicate that this is caused by the high circulating levels of LH (29) present in the blood at this time.

The peak in estrogen level, occurring in the absence of an elevated progesterone concentration, not only initiates the onset of sexual receptivity some 24 hours later, but also results in the ovulation-inducing discharge of gonadotropins from the pituitary at about 4 to 6 hours after the onset of estrus (24, 31, 59, 60). Shortly after this, the ovulatory follicle begins to enlarge from 5 to 12 mm in diameter, which it attains at the time of ovulation (+27 hours). Because the time from the beginning of the rise in peripheral estrogen levels to the onset of estrus is dependent upon the rate of rise and upon the sensitivity of the hypothalamic sex-behavior center to progesterone and estrogen, we must expect breeds of sheep to differ in time with respect to the interval between luteolysis and the onset of estrus. This difference in time to the onset of estrus, by different breeds, may also be reflected in different times between the onset of estrus and the time of the peak LH concentration, hence, the time of ovulation. Minor differences in reported data should be viewed from this standpoint.

The action of estrogen in inducing a sudden release of gonadotropins from the pituitary is mediated or partially mediated through the release of gonadotropin-releasing hormone (GnRH), from the hypothalamus and, or, by enhancing the sensitivity of the gonadotropin-secreting cells of the pituitary to a constant level of GnRH. Experiments on the use of synthetic porcine GnRH in a wide range of animals suggest that both LH and FSH are released simultaneously (33). Although the role of the gonadotropins in initiating or in regulating the growth and atresia of Graafian follicles during the estrous cycle is not known, it is accepted that LH is the normal ovulation-inducing hormone in the ewe; however, synergism with FSH is a possibility. Prolactin also is secreted in large amounts at estrus (16, 34) under the stimulus of estrogen (Fig. 1). Although we have seen that prolactin is required for the subsequent maintenance of the secretory activity of the corpus luteum, the role, if any, of the estrous surge of prolactin in the ovulatory process is not known.

#### IV. Pregnancy

##### A. THE PREATTACHMENT PHASE

Following ovulation the egg passes into the Fallopian tube aided by the movement of the fimbriae of the infundibulum. The part played in this

transport of the ovum by estrogens and  $\text{PGF}_{2\alpha}$  present in the follicular fluid at ovulation is not known.

Fertilization occurs (in the lower region of the ampulla of the oviduct) within hours after ovulation. When the embryo has reached the morula stage, on day 4, i.e., 72 hours after ovulation, it passes through the uterotubal junction into the uterus. An elevated plasma level of estrogen occurs at this time (11, 40, 50, 70), and it is worth speculating on whether this estrogen peak is involved in the transport of the embryo from the Fallopian tube into the uterus.

For the maintenance of pregnancy, the presence of a functional corpus luteum actively secreting progesterone is required. It has been shown that when embryos of ewes are transferred to synchronous recipients at any time up to day 12 pregnancy can ensue, whereas, when they are transferred after this time, the corpus luteum is not maintained beyond day 15, i.e., the time of luteal regression in the normal estrous cycle; consequently, pregnancy does not follow (65). Thus we can say that the maternal organism recognizes the presence of an embryo by, or before, day 12 and by this time initiates some mechanism preventing luteolysis on day 15. The nature of the antiluteolytic and/or luteotropic factor is not known. The transition of the corpus luteum from being one of an estrous cycle to one of pregnancy has led to the concept of the "maternal recognition of pregnancy," but is such a phrase valid? If an antiluteolytic or luteotropic factor is derived from the embryo itself, then the maternal organism is playing a passive or permissive role and does not herself initiate an antiluteolytic process.

The survival of a functional corpus luteum to day 16 of pregnancy, is the basis for the first laboratory pregnancy test in the ewe (61). The index of discrimination between the plasma progesterone concentration of a pregnant, as compared with a nonpregnant ewe, over the period day 16-19 is 4:1. A progesterone level  $>0.8$  ng/ml at this time generally indicates that the ewe is pregnant; the expected level at this stage of pregnancy lies between 2 to 4 ng/ml as compared with  $<0.5$  ng/ml for the nonpregnant ewe.

## B. THE INITIATION OF PLACENTAL ATTACHMENT

In the ewe the union between the embryo and the maternal uterus is a fairly lengthy process. The union is completed when maternal nutrients are supplied to the embryo by attachment sites at which the fetal membrane interdigitates with the maternal uterine caruncles. To summarize, apposition between the uterine luminal surface and the chorionic vesicle engorged with fluid occurs by day 17. This is followed by adhesion be-

tween the chorionic vesicle and the uterine epithelium, and the formation of attachment sites which are first visible around day 25–30 (see Chapter 12).

Classic experiments on implantation in the rat have demonstrated that the ovary plays a dual role in the initiation of embryonic implantation in this species. First, progesterone from the corpora lutea brings the uterus into a receptive state whereas estrogen, primarily from the ovarian follicles, is required to initiate implantation of the embryo.

Experiments on the early removal of the ovaries of the ewe (day 4–7) (4, 23, 47, 68) and the adrenals (12) followed by administration of exogenous progesterone or involving embryo transfer into long-term ovariectomized progesterone-treated ewes (46; Robertson and Smeaton, unpublished data), have demonstrated that in pregnancy ovarian estrogen plays no essential part in initiating placental attachment or in sustaining the subsequent pregnancy.

The finding that in the pig the preattachment chorionic vesicle can synthesize estrogen *in vitro* (52) and the demonstration that estrone sulfate arising from the embryos can be detected in maternal blood of the pig at the time of placental attachment (58) suggests that, as in the rat, estrogen may be involved in initiating a local union between mother and embryo in the sow, and perhaps also in other species such as the ewe. Estrone sulfate has been found in the allantoic fluid of the pregnant ewe at about the time that attachment of the embryonic membranes to the maternal uterine caruncles occurs (8).

Estrone sulfate, which is water soluble, may be imagined as diffusing from the chorionic vesicle at a time when there is tight apposition to the uterine caruncles; at the epithelium of the maternal caruncles the estrone sulfate is deconjugated and reduced to estradiol-17 $\beta$  which initiates the breakdown of the caruncular epithelium. This leads to the formation of attachment sites between the maternal caruncles and the chorionic vesicle, these attachments then developing into fully functional placentomes. The initial production of estrone sulfate, which is biologically inactive, could ensure that only those uterine caruncles in apposition to the conceptus, and not the whole maternal organism, are exposed to active estrogens. As the embryo and its membranes expand and further elongate new attachment sites continue to be formed between day 30–70, during which time high concentrations of estrone sulfate are to be found in the embryonic fluids (8). The drop in the concentration of estrone sulfate that occurs in embryonic fluids about day 60–65 may mark the cessation of formation of new attachment sites, although the sites already formed continue to increase in size for an appreciable time. So far it has not been demonstrated that the preattached sheep embryo synthesizes estrogens, and it may be that the

estrone sulfate is synthesized after the attachments, i.e., the placentomes, are formed. In such a case another hypothesis for the role for estrone sulfate should be sought.

### C. THE MAINTENANCE OF PREGNANCY

For the continuation of pregnancy in the ewe, the presence of an actively secreting corpus luteum is essential during the first 50–60 days. Ovariectomy, enucleation of the corpus luteum, or hypophysectomy, during this period will lead to the termination of the pregnancy. After day 50–60 the activity of the corpus luteum is maintained following hypophysectomy, which suggests that the conceptus may be producing a luteotropin that not only maintains the functional activity of the corpus luteum but may also stimulate increased progesterone synthesis and secretion by the conceptus. This luteotropin may be ovine placental lactogen (oPL) (37). Although the increase in maternal plasma concentrations of oPL parallels the rise in progesterone over the period day 60–110 (37), implying a cause and effect relationship, this may not be the case, as both these increases may be due to the increase in size of the whole conceptus and/or to an increase in size and number of the placentomes. oPL appears to be synthesized by the fetal component of the placenta. Although oPL may afford support for the corpus luteum after day 50–60, the question remains as to why oPL does not provide support earlier when it is known to be actively synthesized as soon as placental attachment has occurred, if not before, i.e., some 30 days earlier (Chan, Robertson, and Friesen, unpublished data). oPL shows no immunological cross-reaction with human or bovine placental lactogens or with ovine prolactin or ovine growth hormone.

As previously stated, steroidogenesis requires the active support of some protein hormone, but what substance stimulates the production of estrone sulfate? It is likely that such a protein is synthesized by the chorion itself, by analogy with human chorionic gonadotropin, if estrone sulfate synthesis does, indeed, precede the formation of the attachment complexes. If estrone sulfate formation begins after attachment has been initiated, then it is more likely that the protein hormone is produced by the attachment complexes. oPL has been shown to be present in the allantoic fluid of the ewe (Chan, Robertson, and Friesen, unpublished data) at the time of attachment and the very close parallelism between the concentration of oPL and estrone sulfate in allantoic fluid from day 30 to 65 implies that the two may be directly related. This is, as yet, unproved.

A steep rise in the concentration of maternal plasma progesterone occurs between day 60–110 and this is due to increased production by the conceptus, and not, as in some species, because of an increase in a progester-

one-binding component of plasma (30). From day 110 until just before parturition the progesterone concentration remains at a level of 12 to 20 ng/ml. During this time the contribution of the corpus luteum to the plasma progesterone pool is very small. It might be assumed that the concentration of progesterone in the maternal plasma during the period between day 100–130 would reflect the number of fetuses or placentas, or both, and that single and twin pregnancies should be readily recognizable. However, although this prediction may be possible statistically, in dealing with large numbers, it is far from reliable on an individual basis. This may be partly because a single fetus may occupy a disproportionately large number of attachment sites in both uterine horns and hence have a greater active placental mass per fetus than in the case of twins. This factor reduces the index of discrimination below the 2:1 ratio for twins versus singles. In addition, biological variation among individuals of a group further reduces the possibility of accurately predicting the number of fetuses in an individual. The rise in concentration of maternal progesterone from 2 to 4 ng/ml at day 60, which is comparable to the peak concentration found during the estrous cycle, to a level of 12 to 20 ng/ml by day 110 enables an accurate assessment of pregnancy to be made based on maternal progesterone concentration during the last trimester of pregnancy even in the presence of cycling nonpregnant ewes. oPL can be detected in serum at about day 30, and its presence can be used as the basis of a specific pregnancy test in the ewe.

#### D. PARTURITION

The ewe has been used extensively as an experimental model for the study of factors involved in parturition (51). This aspect is covered in Chapter 13.

#### E. MAMMARY GLAND DEVELOPMENT AND LACTATION

This area is dealt with in Chapter 14. The ewe should constitute a useful model animal for studying the role of placental lactogens in mammary gland development using purified preparations of oPL.

#### F. SEXUAL ACTIVITY POSTPARTUM

Although lactation depresses ovarian activity during the early postpartum period, the duration of depression being related to the stimulus of being suckled, and to the available dietary intake, the major influence on postpartum ovarian activity in the ewe is that of season. In the majority of breeds, lambs are born during the normal period of seasonal anestrus and

this has led to a great deal of confused thinking in which seasonal anestrus has been ascribed to lactational anestrus.

A small number of ewes (5-15%) will exhibit a postpartum estrus within 36 hours of lambing. In this case, the trigger for the induction of behavioral estrus comes, not from the ovaries, but from the preparturition rise in estrogens superimposed upon a declining progesterone concentration. At this time there are no signs of follicular development in the ovaries and there is normally no ovulation associated with this estrus.

Ewes which lamb during the breeding season and which are not permitted to suckle will generally have ovulated by about day 20 postpartum. Although they ovulate at this time they will not normally conceive should they mate, as the involution of the uterus is not complete and the uterine environment is not normally conducive to the presence of an embryo. Conception would normally occur about day 35 postpartum, i.e., at the next ovulation. When Finnish Landrace ewes were only permitted to suckle their lambs for 24 hours and then were reexposed to rams at intervals of approximately 6 months, i.e., in late summer and again in late winter, over a period of 2.5 years the modal parturition to conception interval was 35 days (78). Although by no means conclusively proven, the onset of postpartum sexual activity may be delayed during the breeding season in lactating ewes. The suppression of ovarian activity in well-nourished ewes lactating during the breeding season is associated not with lactogenesis per se but with the reflex secretion of prolactin from the pituitary brought about by the suckling stimulus (35). Prolactin would appear to be exerting an antigonadal role.

## V. The Exogenous Control of Reproduction of the Ewe

### A. SYNCHRONIZATION OF ESTRUS DURING THE NORMAL BREEDING SEASON

When a flock of ewes are bred during the normal breeding season the overall spread of duration of lambing will be about 40 days if those which do not conceive at their first estrus following exposure to a ram are allowed to be rebred. This time period consists of two estrous cycles (34 days) plus a spread of about 6 days for differences in gestation length. About 70 to 80% of the ewes which lamb will do so from mating during the first 23 days. Under certain conditions of management, it may be desirable to reduce the period of lambing. This can be done by synchronizing estrus and limiting the period during which the ewes are first bred to 1 or 2 days. The length of the estrous cycle is controlled by the functional life span of the corpus luteum, viz, 15 days, and for 12 of the 15 days



the ewe is exposed to elevated levels of progesterone. If during the season of sexual activity the ewe is given exogenous progesterone, or a synthetic progestagen, before the termination of the functional activity of the corpus luteum, then estrus and ovulation will not occur as a consequence of the normal luteolysis of the corpus luteum. These will only occur when the administration of the exogenous progestagen is stopped; estrus occurring at about 36 hours and ovulation occurring in about 60 hours. In a randomly cycling flock of ewes the exogenous progestagen treatment must last for a period of 15 days to ensure that the estrus of all animals is synchronized. The most practical methods for administering the progestagen are by the use of impregnated vaginal sponges or silicone implants inserted under the skin. Both of these methods permit a slow steady release of the progestagen.

There is one main problem associated with the synchronization of estrus. The level of fertility is lower than normal and this has been shown to be associated with a depression of fertilization due to impeded sperm transport. Once ewes have been synchronized they usually remain well synchronized through the second estrous period so that treatment can be scheduled to breed the ewes at the second rather than at the first estrous period thus avoiding the problems of lowered fertility. To be effective, the level of exogenous progestagen given must be high enough to prevent ovulation and that will be approximately equivalent to the level of progesterone found at midcycle. It can be seen that in the extreme case of a ewe which has just ovulated before the progestagen treatment is commenced, its uterus will be subjected to the effects of both the endogenous progesterone and the exogenous progestagen, i.e., double the normal concentration for about 12 days. This, no doubt, affects the cervical and vaginal secretions in such a way as to impede sperm transport. Similarly, a ewe which is just about at the end of the cycle when the progestagen treatment is commenced will have been exposed to a progestagen for twice the normal duration and this may again contribute to a lowered pregnancy rate. It should be realized that if successive groups of a large number of ewes are synchronized to come into estrus over a protracted period of time, a correspondingly large number of rams must be available. When the number of rams is limited this will result in a decrease of sperm numbers in the ejaculate thus reducing the possibility of fertilization in a situation in which sperm transport is impeded (63).

#### B. INDUCTION OF FERTILE MATINGS DURING ANESTRUS

If the ewe is to conceive from a natural mating she must experience estrus followed by ovulation. The anestrus ewe is first preconditioned

with a progestagen given via a vaginal pessary or implant maintained for 12 days. Pregnant mare serum gonadotropin (PMSG 450 IU) is given intramuscularly at the time of progestagen withdrawal. The ewe is sexually receptive about 36 hours later and ovulates 60–66 hours after the PMSG injection. However, the time to estrus is variable depending upon the half-life of the progestagen used, the dose given, and the breed of sheep. Pregnancy rates following such a treatment are variable, ranging from 30 to 70%, although normally a high percentage of the ewes are sexually receptive and are bred. A possible confounding of these results, due to a decrease in libido and sperm production of the ram at this season of the year, should not be overlooked. It should be noted that although PMSG has both FSH and LH properties, its role is to stimulate follicular development which gives rise to an estrus-inducing peak of estradiol-17 $\beta$  in progestagen-primed animals. This estradiol-17 $\beta$  peak then leads to the ovulation-inducing discharge of LH and FSH from the ewe's own pituitary, i.e., PMSG does not induce ovulation and luteinization directly. At a dose level of 450 IU of PMSG, superovulation does not normally occur although some slight increase in ovulation rate is seen.

### C. ARTIFICIAL INSEMINATION

The use of AI in sheep has been largely confined to the Soviet Union and to eastern and central Europe, i.e., areas of low labor costs. Russian workers break their flocks down to units of 2500 and use vasectomized rams to detect estrus. As ewes are detected in estrus (45) they are inseminated with raw semen collected by an artificial vagina from rams allocated to the unit, the dilution rate being about 1:6 with a conception rate of 50 to 90% for a single insemination (39). The synchronization of estrus, with or without ovulation induction with PMSG, and AI are complementary in the ewe. On the one hand, the cost of maintaining the large number of rams required to mate a synchronized flock is prohibitive while the labor costs of AI in a nonsynchronized flock is again prohibitive. The two used together may be economical. For optimum management efficiency, a single insemination at a fixed time relative to the time of ovulation must be used. One of the main pitfalls to success in the use of AI is the optimum time of insemination relative to the time of ovulation. There are differing opinions on this topic with recommendations when using diluted fresh semen, ranging from "as soon as the ewe is found in estrus" to 12–14 hours after estrus is first observed, i.e., 16 hours before ovulation. It is therefore important to know when ovulation occurs relative to the particular treatment being used, and to adjust the time of insemination accordingly. This time to ovulation will vary slightly between breeds. As a

It should be noted, however, that after an initial decrease during the first 40 days of pregnancy the luteal cells of the corpus luteum of the pregnant goat decrease in size and then increase until day 110 before a slow decline to parturition sets in. The secretory activity of the corpus luteum tends to follow the change in the size of the luteal cells with the peripheral plasma progesterone concentration reaching its maximum about day 110 (77). Since hypophysectomy in the goat will lead to abortion (10) it has been concluded that the conceptus does not produce a luteotropic factor, but this may be a matter of degree. The increase in size of the luteal cells over the period 40–110 days may arise through stimulation by some placental luteotropin. The presence of a placental lactogen in the pregnant goat has been reported (7). The incidence of pseudohermaphroditism is probably higher (6–15%) in dairy goats than in any other farm animal (20, 21).

Goat semen can be frozen and successfully used for artificial insemination. If goat semen is diluted with an egg yolk extender the sperm become immobilized after a short period of time (66). A similar effect is observed if lysolecithin is added to goat semen and the inference has been made that this substance, toxic to goat sperm, is formed when goat semen is stored in an egg yolk-containing diluter (1).

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# 19 Reproduction in the Dog and Cat

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## I. Estrous Cycle

### A. PUBERTY

Puberty usually occurs at 6 to 12 months in the dog with the female reaching sexual maturity several weeks prior to the male (66). Dogs usually become sexually mature within 2 or 3 months after reaching their

adult body weight (2) As smaller breeds reach adult body size at an earlier age, it follows that small breeds usually experience puberty earlier than larger breeds Puberty may be somewhat dependent upon the environment since free roaming animals become sexually mature sooner than kenneled animals (43)

The onset of puberty in the female cat is influenced more by the time between birth and the onset of the breeding season than by age Kittens born from October to December may not become sexually mature by the next sexual season which starts in January, and thus may be 12-16 months old at first estrus This explains the large range of age at first estrus reported for cats, namely, 6-8 months with an average onset of 11.6 months (59) These findings are similar to those reported by Klug (61)

## B SEASONAL INCIDENCE

Although many breeders believe that there are two breeding seasons a year for the dog, examination of American Kennel Club records fails to show a bimodal curve for onset of estrus Asdell (3) and Christie and Bell (13) likewise did not find a bimodal distribution of estrous periods in a study of 1561 cycles, although more cycles occurred during the first half as compared to the latter half of the year (Fig 1) It is also of interest that estrous cycles occur during all seasons of the year Thus the use of the term monestrous is appropriate for describing the occurrence of estrus in the dog

A seasonal incidence of litters has been reported for the cat with two peaks noted, one in the spring and one during mid- or late summer

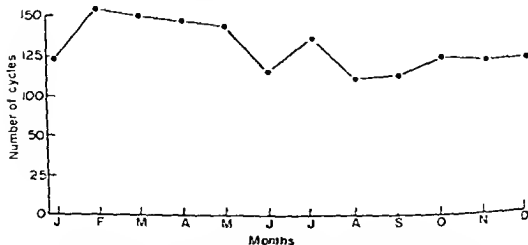


FIG 1 Frequency distribution of the time of onset of proestrus in the dog during the months of the year Data are from Christie and Bell (13)

(75, 82). The timing of this bimodal incidence of litters is probably due to the duration of gestation in the cat (approximately 2 months) and the occurrence of an anestrus period during the fall and early winter. Fertile matings in January would result in litters being delivered in March with rebreeding occurring again 4–6 weeks later with the subsequent delivery of another litter in midsummer. Although some cats have three litters a year, the average is closer to two per year (75).

### C. TERMINOLOGY

The terminology originally developed for the dog included anestrus, proestrus, estrus, and metestrus. Metestrus has been used to indicate both metestrus and diestrus, as used in other animals having estrous cycles. In addition, metestrus in the dog has been further divided into metestrus I and II, i.e., the first and second halves of the luteal phase. The problem of terminology is due, in part, to the fact that the bitch remains sexually receptive for a number of days after ovulating and at a time when newly formed corpora lutea are developing both structurally and endocrinologically, in contrast to the large domestic animal species in which sexual receptivity usually terminates within hours after ovulation. Holst and Phemister (51) observed a sharp decline in cornified cells from the vaginal epithelium about 3 days before the end of estrus. They have suggested diestrus be used for the luteal phase of the cycle and that this phase begin the first day there is a significant decline in the cornified cells, accompanied by a concurrent rise in noncornified cells, as seen in the vaginal smear. The suggestion has also been made that the term metestrus be used to designate not only the time of the cycle involving luteal activity but also the time required for tissue repair following progestational stimulation (55). The authors feel the simplest approach is to recognize that metestrus (early luteal function) occurs during the latter part of estrus in the dog (51) and to use the term diestrus for the luteal phase of the cycle beginning with the loss of sexual receptivity.

The terms anestrus, proestrus, estrus, and metestrus have been used to describe the estrous cycle of the cat. The use of the term metestrus should be confined to the early developmental stages of the corpus luteum as used in other species. As will be discussed later, corpora lutea are only present in nonpregnant animals if a sterile mating occurs. The cat is similar to the dog in that the female stays sexually receptive for a few days after ovulation, even in the presence of active corpora lutea (71). Thus metestrus (early luteal function) occurs within estrus as for the dog. The term diestrus would be preferable for the period during which corpora lutea are functional in the nonpregnant cat.



The cat undergoes periods of ovarian quiescence, anestrus, during the fall and early winter, with resumption of cyclic ovarian activity beginning often in January in the temperate zone.

#### D. DURATION

The original concept of two estrous cycles a year for the dog appears to be erroneous as most reports indicate an average interval of 7 and 8 months (2, 13). There is a tremendous variation in the duration of estrous cycles with a range of from 16 to 56 weeks reported (13) (Fig. 2). There appears to be less variability within animals than within the breed. Although significant breed differences are not the rule, some breed differences are significant, i.e., 26 weeks for the Alsatian dog versus 33.5 weeks for the standard dachshund (33). The Basenji is an exception to the average 7-month interval in that only one estrus occurs each year, usually in the fall (33). The condition appears to be caused by a recessive gene (78).

Christie and Bell (13) found no relationship between breed size and estrous cycle interval. They did observe that pregnant bitches had significantly longer intervals (32 weeks) between heats than nonpregnant dogs

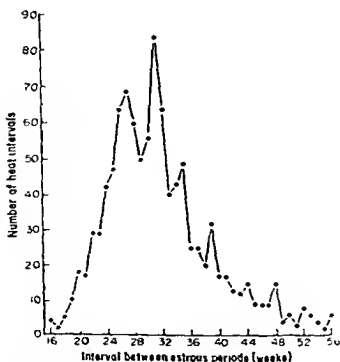


FIG. 2. Frequency distribution of the duration of estrous cycle intervals in the dog. Adapted from Christie and Bell (13).

(29 weeks). Similar findings were reported by Coneannon, Hansel, and Visek (18). These reports are in contrast to an earlier one in which non-bred dogs had estrous cycle intervals that were 16 days longer than those of pregnant animals (2).

The authors have observed a tendency for older dogs to have increased intervals between estrus. There does not, however, seem to be any reduction in the conception rate as a result of the prolonged cyclic intervals.

Each component of the estrous cycle of the dog is relatively long in duration as compared to the large domestic animals. The generally accepted values include: 9 days for proestrus (range 3–16), 9–10 days for estrus (range 4–12), 75 days for diestrus (range 51–82), and 125 days for anestrus (range 15–265) (2, 18, 27, 85). The greatest source of variation in the duration of the estrous cycle length is due to anestrus, although the length of diestrus is very dominant in the dog.

The length of the estrous cycle in nonpregnant cats is dependent upon coital contact with a male. In anovulatory cycles, the intervals between the beginning of successive estrous periods are variable, although they are often 2–3 weeks in duration (71) (Fig. 3). The estrous cycle intervals in cats that ovulate but fail to conceive is longer, averaging about 6 weeks in duration with a range of 30 to 75 days (31, 71).

Proestrus is 1–3 days in length in the cat (69). The duration of estrus appears to be influenced by the occurrence of ovulation, in that ovulating cats had estrous periods of 5.7 days whereas the same cats had an average interval of 8 days if ovulation did not occur (71). These results agree with previous reports (63, 82).

## E. OVARIAN FUNCTION

An extensive presentation of the morphology of the canine ovary can be found in the recent book of Andersen and Simpson (1).

### 1. Folliculogenesis

The follicular phase of the estrous cycle of the bitch is unique because of its length—an average of 13 to 14 days being required for follicular development prior to ovulation. Effects of estrogens secreted by the follicles are manifested within several days after the start of follicular growth by vulval swelling and discharge from the external genitalia. This period (proestrus) is unique in the dog because of its length and because of the distinct genital tract changes associated with it.

Follicular development leading to the occurrence of the first estrus and ovulation at puberty in the dog appears to start abruptly without previous

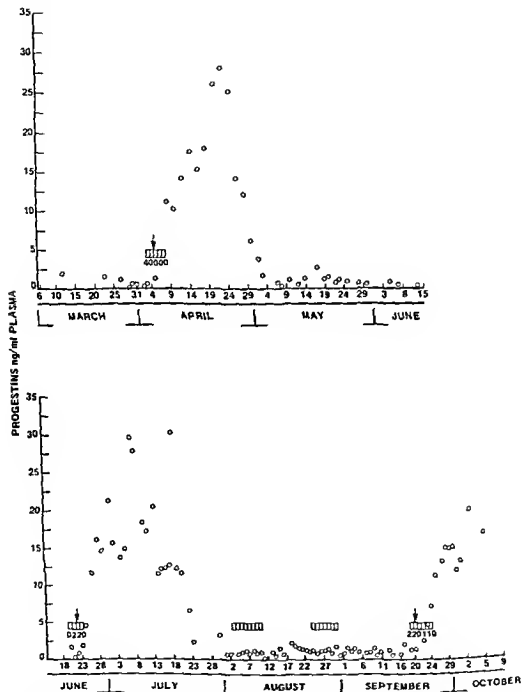


Fig 3 Luteal activity in a cat during nonfertile ovulatory and anovulatory cycles. No coital contact was allowed with a male during August. The horizontal boxes represent the days of sexual receptivity as determined by behavior of the female. The number of copulations each day of estrus are indicated below the horizontal boxes. The estimated day of ovulation is indicated by an arrow. From Paape *et al* (71)

anovulatory waves of follicle growth (58). This is also probably true in sexually mature animals.

The follicular phase of the estrous cycle of the cat lasts 5–6 days if ovulation occurs. While the growth period prior to the onset of proestrus is relatively short (1–2 days) as in the dog, proestrus is much shorter in the cat (2 days). The reason for the longer period of estrogen secretion in the dog prior to the ovulatory release of gonadotropins as compared to the cat is not apparent at the present time. If an ovulatory coital stimulus does not occur, follicles tend to undergo periods of growth, maintenance, and regression over 10- to 12-day intervals in the cat. A new wave of follicles begins within a few days with the recurrence of estrus soon after. Considerable variation in the duration of estrus in nonovulating cats exists with some animals remaining sexually receptive most of the time suggesting that continual patterns of follicle growth occur in some individuals.

## 2. Ovulation

Andersen and Simpson (1) and Smith and McDonald (85) have reported the occurrence of ovulation during the first day of estrus. Phemister *et al.* (72) found most ovulations (84%) occurred on days 1–4 of estrus with the mean value being day 2.9. They did, however, report a wide range of values with ovulation occurring as early as 2 days before the onset of heat and as late as 7 days after onset. Andersen (personal communication) has suggested that younger bitches may ovulate earlier in the estrous period than older, more experienced females. More experienced dogs may be behaviorally more responsive to estrogen and show sexual receptivity earlier in relationship to the increase in levels of estrogen. As a result, ovulation would occur later in relation to the onset of sexual receptivity. The variation in the time of ovulation as compared to the onset of estrus is undoubtedly a function of the variability of the onset of sexual receptivity since the endocrine events that precede ovulation appear to be relatively uniform in their temporal relationship. It is likely, however, that ovulation occurs around the time of first acceptance of the male and that failure to observe this in some studies may be the result of males failing to show interest because of preferential breeding habits (5).

The cat is an induced ovulator requiring a coital stimulus for ovulation. While a single coital stimulus may be sufficient for some cats (71), it is apparent that repetition of the coital stimulus may be a prerequisite for ovulation in others (36, 64, 82). Recently the authors (unpublished observations) have observed ovulatory failure following three sexual contacts within a 30-minute period. As pointed out by Greulich (36), the onset of sexual receptivity in the cat may not always coincide with the

requisite ovarian conditions necessary for ovulation. In other words, sexual receptivity may occur before follicles can respond to the gonadotropin surge which presumably precedes ovulation in the cat.

The coitus-to-ovulation interval in the cat is quite variable with a range of 25 to 50 hours reported (36, 64). More recently, Paape *et al.* (71) reported an average interval of 45 hours; this is probably an overestimation of the interval. It remains to be tested as to whether or not this variability is due to variations in follicle maturation at the time of coitus or variations in the intensity of the coital stimulus, or both. Ovulation has been induced in cats by stimulation of the vaginal tract by a glass rod (36). In the authors' experience, the stimulation of ovulation by artificial means requires sufficient penetration of the vaginal tract to cause the typical postcoital reaction of the female.

The endocrinology associated with follicular development and ovulation in the bitch is similar to that reported for other species (Fig. 4). The increased estrogen production during proestrus is followed by an ovulatory surge of LH at the beginning of estrus (18, 52, 57, 70, 85). The ovulatory

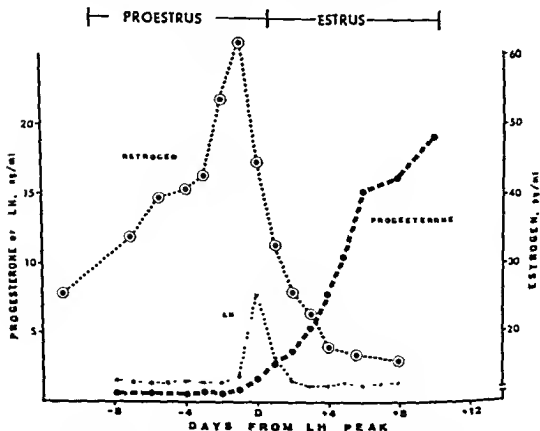


FIG. 4 The endocrine and behavioral aspects of the periovulatory period in the dog. Courtesy of Concannon *et al.* (18).

surge of LH appears to be of longer duration than for most other species in that LH values have been reported elevated over basal levels for as long as 48 hours. The LH surge is closely related to the onset of sexual receptivity as LH peaks were observed either on the last day of proestrus or the first day of estrus (18, 85), while Phemister *et al.* (72) reported that the interval between estrus and the LH surge is close to 48 hours.

The interval between the first and last ovulation in the dog has been reported to extend over a period of 12 to 72 hours (37). However, other reports indicate that ovulation of all follicles is almost simultaneous taking only a few hours for completion (1, 27). The latter possibility appears to be the most likely pattern of ovulation.

The authors are not aware of any endocrine data concerning the pre-ovulatory and ovulatory events in the cat.

### 3. Luteal Activity

The dog is unusual in that luteal activity is essentially of the same duration whether or not pregnancy occurs. The typical pattern of luteal function in the nonpregnant bitch is as follows: peak luteal activity occurs at about 20 days postovulation and is followed by the establishment of a transient plateau. This plateau is followed by a prolonged decline in luteal activity with regression being completed by about the 75th–80th day postovulation (15, 18, 85, 91) (Fig. 5).

Corpora lutea in the nonpregnant cat are formed only after nonfertile coital stimulation. When luteal function was studied following nonfertile

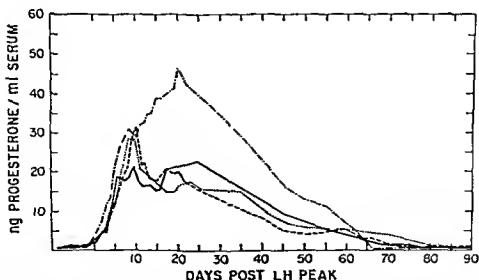


FIG. 5. Luteal activity (progesterone levels) in nonbred (----), sterile-bred (....), pseudopregnant (—), and pregnant (-.-.-) dogs. The data are from Smith and McDonald (85).

coitus, the average life span of the corpora lutea was 36.5 days (71). Peak luteal activity occurred by day 16 or 17 postovulation and was followed by a plateau of luteal activity for about 1 week. Gradual regression of the corpora lutea then occurred over a 2-week period (to day 36 postovulation) (see Fig. 3).

A graphic summary of the relationship between sexual receptivity, ovulation and corpus luteum life span is shown in Fig. 6.

## F TUBULAR GENITALIA

Sokolowski *et al.* (88) have reported in detail the gross and microscopic changes of the reproductive tract of the bitch during the estrous cycle.

The tubular genitalia of the dog are very responsive to the endocrine changes which occur in association with follicle growth and luteal activity. The external genitalia begin to enlarge within a few days after follicular growth begins. This enlargement and the increased discharge from the vulva are the main signs which signal the onset of the cycle. The main changes that occur within the tubular system involve hyperplasia of the epithelium of the vagina, growth of the endometrium, and bleeding from the endometrium.

The uterus of the bitch rapidly increases in size during the early part of the luteal phase of the cycle due to the proliferation of glandular elements. A corkscrew-type appearance has been ascribed to the uterus at this time (88).

The uterus of the dog appears to be unusual in its exaggerated response to progesterone. Uterine involution is not complete in the nonpregnant dog until 120–150 days postovulation and, often, not until after 150 days postovulation in the pregnant animal (188) (Figs. 7 and 8). This prolonged involutionary period probably is associated with the prolonged luteal phase of the cycle coupled with an apparent genetic sensitivity to progesterone. These prolonged periods of endometrial progestational stimulation normally produce glandular hyperplasia and minor cyst formation. Repeated cycles of hyperplastic change in the endometrial glands may lead to a persistence of these changes beyond the usual recovery period of the endometrium. This, in turn, can establish an environment which is conducive to the development of endometritis and pyometra (22).

During proestrus, growth of the vaginal squamous epithelium with cornification of the superficial layers occurs in response to the rising follicular estrogens. This thickened epithelium presents a barrier to the movement of neutrophils into the vagina. The proliferation of the endometrial vascular system during estrogen induced endometrial growth allows the diapedesis of red blood cells into the uterine lumen. Thus the uterus is the

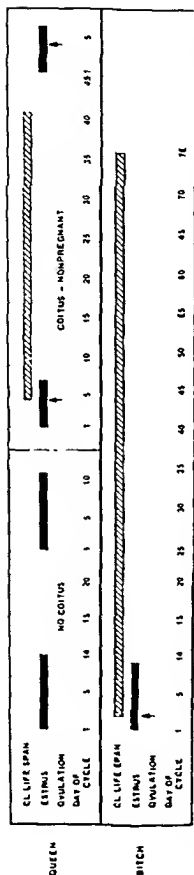


FIG. 6. A comparison of the relationship of sexual receptivity, ovulation, and corpus luteum activity in the nonpregnant dog and cat. The graph is from Stabenfeldt (89).





FIG 7 (A) The endometrium of a dog 120 days after the onset of estrus. Clumps of exfoliated epithelial cells are still common in the lumen of the uterus (arrow)  $\times 35$  (B) A higher magnification of the same section showing that epithelial lining is still irregularly arranged and partially denuded areas exist (arrow)  $\times 275$ . The photomicrographs are from Andersen and Simpson (1)

source of the red blood cells that appear in the vaginal smear. Cornification of epithelial cells is maximal at the beginning of estrus. The vaginal epithelium is maintained in a state of cornification during declining estrogen levels for at least 5 days after ovulation. As estrogen levels diminish and progesterone levels rise after ovulation, the appearance of the exfoliated vaginal epithelial cells changes dramatically within 1 or 2 days. Deprived of estrogenic stimulation, the vaginal epithelium diminishes in thickness and noneornified cells reappear. Neutrophils again are able to penetrate the epithelium and thus appear in great numbers during late estrus and early metestrus. Endometrial capillary growth ceases and with the cervix closed, the red blood cells do not appear in the vaginal smear.

Gross changes of the external genitalia of the cat are not as marked as in the dog. The amount of swelling associated with the external genitalia and the amount of discharge are considerably less in the queen. However, the sequence and degree of endometrial change are similar to those described for the bitch (28).

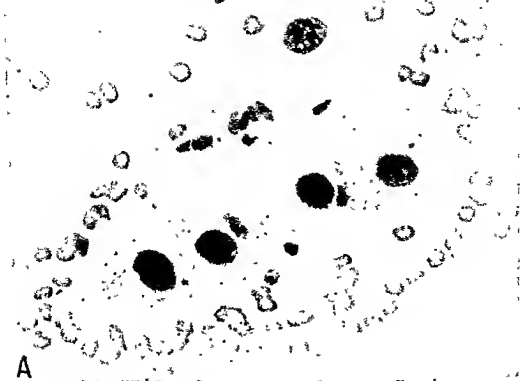
Changes in vaginal exfoliative cells can be used to determine the stage of the estrous cycle of dogs and cats with considerable accuracy (14, 69,



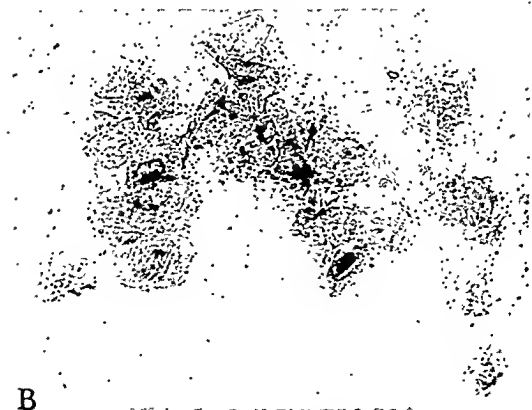
FIG. 8. (A) The endometrium of a dog 138 days postcoitum (10 weeks postpartum). The endometrium has become reduced in thickness and sloughing is diminishing. The arrow points to a uterine gland shown in (B).  $\times 30$ . (B) A higher magnification of the same section showing desquamation of surface epithelial cells within a uterine gland. Plasma cells and macrophages are present in the stroma.  $\times 250$ . The photomicrographs are from Andersen and Simpson (1).

82) (Figs. 9 and 10). Schutte (76) has developed a procedure which differentially determines the proportion of exfoliated cells termed anuclear, superficial, large intermediate, small intermediate, and parabasal cells as a function of the stage of the cycle. Christie *et al.* (11) have simplified Schutte's classification by adopting the method of Wachtel (94) which recognizes the intermediate cells as one type of cell. The main changes observed are a rise in the proportion of anuclear and superficial cells during proestrus and estrus, and an increase in the number of intermediate and parabasal cells at the beginning of diestrus. The "eosinophilic index" has also been used as an indicator of the stage of the cycle (14). The index refers to the percentage of cells (excluding parabasals) that stain orange when exposed to the Schorr trichrome stain. The eosinophilic index increases rapidly during proestrus becoming as high as 90% at the end.

Although some investigators have placed less emphasis on the value of vaginal smear cytology in the bitch, the technique is valuable for assessing the stage of the estrous cycle, particularly in reference to ovulation. Peak cornification of the vaginal epithelium usually occurs on the



A



B

FIG. 9. Appearance of exfoliated vaginal cells in the dog.  $\times 100$ . (A) Vaginal smear in proestrus. Erythrocytes and noncornified cells predominate. (B) Vaginal smear in estrus. Cornified cells predominate.



FIG. 10. Exfoliated vaginal cells from a cat in early estrus. Superficial cells are distinguished from intermediate cells by presence of small pyknotic nuclei. Courtesy of S. Paape.  $\times 250$ .

first or second day of estrus. Thus if sequential vaginal smears have been obtained, the time of ovulation can be predicted to be close to the first time that vaginal smear cytological changes show only anuclear and superficial cells. A number of cases have come to the authors' attention of apparent reproductive failure in which animals were presented as just becoming sexually receptive, whereas vaginal smear cytological examination and peripheral blood analysis for progesterone (verification of ovulation) revealed that ovulation had occurred several days previously and that the onset of sexual receptivity had not been accurately determined. Instances of prolonged proestrus followed by normal sexual receptivity and ovulation also have been observed by the authors and others (97). The use of vaginal smear cytology is obviously also of benefit in these cases. It is worth emphasizing that ovarian and endocrine changes proceed concomitantly and that the progression of these events is reflected quite well in the cytology of the vaginal smear.

The use of the vaginal smear in the cat to predict ovulation is not valid because the cat is an induced ovulator; however, it may be used to confirm behavioral estrus. Vaginal smear cytology is usually used as a research tool in the cat.

## G. PSEUDOPREGNANCY

Pseudopregnancy is often used in conjunction, or synonymously, with the luteal phase of the estrous cycle of the nonpregnant dog. Some animals will show a gradual deposition of abdominal fat, mammary gland development, and may even manifest mothering tendencies (nest building and/or lactation) at approximately 60 days postovulation (96). There is considerable variability among dogs as to the intensity of these manifestations during the luteal phase with minor changes occurring in most of affected animals. The incidence of clinical signs of pregnancy in nonpregnant dogs is probably low, as Andersen and Wooten (2) have reported a 3% incidence in normal virgin beagles maintained in a large colony. Fidler *et al.* (29) found an incidence of 19.5% of the condition in 245 normal estrous cycles. Frost (32), however, observed an incidence of up to 40% in a series of dogs he examined. The authors feel the term pseudopregnancy or false pregnancy should be reserved for only those nonpregnant animals that manifest obvious clinical signs of pregnancy.

The basis for the development of signs of pregnancy in the absence of a developing fetus is most likely endocrinological in origin. Smith and McDonald (85) have shown that progesterone secretion *per se* is not the cause of lactation in nonpregnant dogs in that progesterone levels in these animals are equivalent to those in nonpregnant, nonlactating dogs at all stages of the estrous cycle (Fig 5). The role of prolactin in this syndrome awaits investigation.

Pseudopregnancy occurs in the cat following ovulation coupled with fertilization failure. Foster and Hisaw (31) presented evidence to show that the pseudopregnancy in the cat lasted for about 6 weeks. More recently, Paape *et al* (71) reported that the average interval between estrous periods is 41 days in pseudopregnant cats (range, 30–73 days). In this study, most cats showed signs of sexual receptivity within a week after regression of the corpora lutea (approximately day 36 postovulation). The signs of pseudopregnancy are considerably less pronounced in the cat than in the dog.

## II. Pregnancy and Parturition

### A. GESTATION AND LITTER SIZE

Failure to determine the exact time of ovulation and fertilization makes it difficult to state precisely the duration of gestation in the bitch. Holst and Phemister (51) found an average interval from breeding to parturi-

tion of 63.6 days with equal numbers of animals delivering on days 62–66 of gestation (range, 59–68 days).

The gestation period of the cat is almost identical to that of the dog in terms of average length (63 days) and variability (58–65 days) (66).

Litter size in domestic dogs varies considerably. Larger breeds often will have eight to twelve pups (highest recorded number = 23) while toy breeds often average one to three pups per litter (10). The average litter size in colony and free-living cats in the United States was found to be 4.03 and 3.88, respectively (81), and 4.8 in colony cats in Germany (59).

## B. FERTILIZATION

The time of fertilization of the ova in the dog appears to be later than previously thought. Phemister *et al.* (72) presented evidence that the canine ovum may require 2 or 3 days following ovulation before it can be fertilized. This is probably due to the fact that the dog is one of the few species in which ova are released prior to the formation of the first polar body, i.e., released as primary oocytes (64).

The longevity of spermatozoa in the reproductive tract of the female dog is striking as compared to many mammalian species. Doak *et al.* (21) found undiminished concentrations of motile spermatozoa in the uterus for up to 6 days postinsemination and in lesser amounts for 11 days postbreeding. In addition, Holst and Phemister (51) have shown high levels of fertility are attained by a single breeding over a range of 3 to 10 days prior to the onset of diestrus. Thus it appears that a major portion of the apparent longevity of canine germ cells, in general, comes from the ability of spermatozoa to survive for long periods of time in the uterus.

The time limits for fertilization of the ovum of the cat have not been established, although it is likely to be similar to most mammalian species (12–24 hours) in that the ova are in a more advanced stage of development as compared to the dog at the time of ovulation (first polar body already formed). The duration of viability of spermatozoa also is not established, but it is likely to be at least as long as the interval from ovulatory coitus to the time of ovulation (24–48 hours).

## C. OVIDUCTAL TRANSPORT

A range of 4 to 10 days has been reported for the interval between the time of coitus and the entry of zygotes into the uterus of the dog (26, 50). Because of the period of time required for oocyte maturation prior to fertilization, it is probable that this is an overestimation of the time required

for oviductal transport in the dog. The duration of oviductal transport in the cat has been reported to be a 4-5 days postcoitum (3, 49) or 2-4 days following fertilization. The administration of estrogens during the time of oviductal transport can interfere with fertility in both the dog and the cat (49, 60) through retardation of zygote transport and development. The temporal relationships during gestation in the dog and cat are shown in Table I.

#### D. ENDOCRINOLOGY OF PREGNANCY

Both the dog and the cat are dependent upon the ovary for the maintenance of pregnancy for most, if not all, of gestation. Sokolowski (87)

TABLE I

Temporal Relationships in Pregnancy of the Dog and Cat\*

Event	Time (days)
1. Ovum in oviduct	1-8 (dog and cat)
2. Morula in oviduct	5-12 (dog)
3. Free-floating blastocysts in uterus	6-20 (dog)
4. Implantation, primitive streak formation	12-15 (cat) 17-21 (dog)
5. Uterine enlargement first seen on radiographs	21 (cat)
6. Pregnancy palpable through abdomen, swellings are first pear-shaped, then spherical, finally ovoid	21-35 (dog)
7. Canine body characteristics recognizable, eyelids developing, sex determination possible. Uterus folding over, may cause discomfort. Female shows lowered hemoglobin, leukocytosis, and increased sedimentation rate	35 (dog)
8. Uterus obviously enlarged	35 (cat)
9. Eyes closed, lids fused, claws on digits, hair begins to grow. Scrotal and vulval tissue prominent, color markings appear	40 (dog)
10. Partial ossification first observable on radiographs	40 (cat)
11. Ossification recognizable on radiographs. Mammae begin to grow and feel turgid	45 (dog and cat)
12. Pregnancy again palpable through abdominal wall, radiographs more reliable, ultrasound reliable for pregnancy diagnosis	50 (dog)
13. Fetus 102 mm in size	50 (cat)
14. Radiographs may be used to measure size of fetuses. Haircoat is developed	55 (dog)
15. Dam shows gradual drop in body temperature until lowest point (98°-99°F) is reached about 24 hours before parturition	about 63 (dog)

\* Adapted from Boyd (9), Evans (26), Holst and Phemister (50), Lamm (62), and Tiedeman and Henschel (92).

\* Day 1 is day of coitus.

found that ovariectomy in the dog as late as day 56 postbreeding resulted in premature termination of gestation. The cat may be less dependent upon the ovary in the latter part of gestation in that it has been found that ovariectomy before day 46 of pregnancy, but not after day 49, will cause abortion (38) as quoted by Asdell (4).

An enhancement of luteal function has been reported for the pregnant dog as compared to the nonpregnant dog beginning at about 15 days post-ovulation (85). This increase in progesterone secretion suggests the possibility of a placental luteotropin as implantation begins at about this time (50). Other workers, however, have not found an enhancement of luteal activity in the pregnant dog (18, 40). While Concannon *et al.* (18) have found higher levels of estrogens in pregnant versus nonpregnant dogs, others have found no difference (24, 39, 40).

The termination of pregnancy in the dog is preceded by the regression of corpora lutea and a decline in progesterone has been noted about 24 hours prior to delivery (18, 40, 85). Thus luteal function in the dog is longer in nonpregnant animals than in pregnant ones.

## E. PARTURITION

The normal sequence of events at parturition is somewhat similar in the dog and cat. Parturition is preceded by an increase in restlessness and a decrease in appetite. In the dog, there is a decided drop in rectal temperature that reaches its lowest point about 24 to 48 hours before delivery. In both species, nesting behavior is intensified and antagonism to strangers is increased just prior to delivery. Also at this time the vulva becomes relaxed and clear mucus may be passed. Uterine contractions are manifested in the dog through sharply increased panting and bodily twitching. As the head of the fetus engages the pelvic canal, abdominal contractions commence. Delivery usually follows three to five contractions.

There can be considerable variation between the delivery of individual fetuses in both dogs and cats with intervals as short as 5 minutes and as long as an hour (19, 66). Most of the time involved in the longer intervals between delivery is spent resting or taking care of any newborn while the actual delivery process including uterine contractions require a relatively short period of time. Both the dog and the cat ingest the fetal membranes following delivery, most of the time without any apparent ill health as a result. This, however, has not been proved to be a source, essential or otherwise, of hormones or nutrients for the dam.

Pregnancy diagnosis is usually accomplished in the dog and the cat by palpation of the fetuses between 26th and 30th day postcoitum. Reliable radiographic evidence of pregnancy may be obtained at 45 days in the cat



(9, 92) and 50 days in the dog (74). Ultrasonic diagnosis has been used at 30 to 35 days (47) and 50 days (62) in the dog. Chemical means of pregnancy diagnosis have not been successful in either species.

## F. POSTPARTUM OVARIAN ACTIVITY

As indicated previously, ovarian activity is not reestablished in the dog until 5 or 6 months postpartum. Also, pregnancy appears to increase the length between estrous cycles (13, 18).

Resumption of ovarian activity appears to occur sooner in the cat during the postpartum period. Although Longley (64) suggested that the newborn had to be removed soon after birth in order for ovarian cyclicity to be reestablished within 4 to 6 weeks, Scott (80) observed cyclic ovarian activity at this same time in spite of the nursing of young.

## III. Sexual Behavior

### A. DOG

Just prior to the onset of proestrus, females may show behavioral changes such as increased appetite and preferred association with males. Christie and Bell (12) reported a continuous increase of interest of the female in the male during proestrus culminating in full sexual receptivity a week after the onset of proestrus. Concannon *et al* (18) described a slightly different pattern in which the dog in proestrus progressively moves from resistance to passivity. The onset of estrus is marked by a clear change in attitude of the female dog with lateral deviation of the tail and presentation of the vulva to the male. Concannon *et al* (18) have suggested that variations in criteria used to determine the onset of estrus are probably responsible for some of the variation reported in the correlation between endocrine events and behavioral estrus in the dog.

Estrous female dogs show definite preferences for certain males (5). Although variation in this selectivity was noted among females, all showed some degree of discriminatory response. Knowledge of preferential response to males is important for breeders to understand so that proper adjustments in pairing can be effected if necessary.

The endocrinological aspects of sexual receptivity in the bitch are interesting, both from the aspect of initiation and maintenance. Several workers have presented data that suggest luteinization of the follicles and progesterone production begins several days prior to ovulation (18, 39, 72). The initiation of sexual receptivity in the dog may require the presence of small amounts of progesterone as well as the presence of estrogen. The prolonged period of sexual receptivity in the dog following ovulation is puzzling be-

cause estrogen levels decline continuously throughout estrus and reach basal levels several days before the end of sexual receptivity (39).

Copulation in the dog is unique because the male becomes locked inside the female following intromission of the nonerect penis, which is stiffened by the os penis. The male is able to dismount during ejaculation, remaining tied to the female tail-to-tail for up to 30 minutes until detumescence of the penis occurs. Dogs should not be forcibly separated during this time since severe damage to the external genitalia may result. Coital stages involved in this process are depicted in Fig. 11. The stages include mounting and intromission, followed almost immediately by the intense ejaculatory reaction which lasts 15–30 seconds. This is manifested by rapid pelvic movement and alternate stepping with the hind legs. During this time, rapid vascular engorgement of the bulbus glandis occurs (46). The bulbus glandis and pars longa glandis are held within the vagina by the contracted vestibulovaginal sphincter of the female. When the male dismounts during the genital lock, the dorsal surface of the penis remains dorsal within the vagina and the pars longa glandis elongates substantially, placing the opening of the penile urethra next to the anterior fornix of the vagina and the cervical os. This facilitates entry of the ejaculate into the uterus (35). ✓

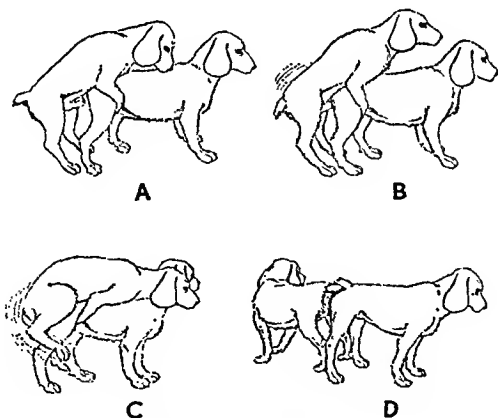


FIG. 11. Different stages in mating of the male dog: (A) mounting and clasp; (B) pelvic thrusting; (C) intense ejaculatory reaction; (D) copulatory lock. The drawing is from Harl (45). Copyright 1967 by the American Psychological Association. Reprinted by permission.

## B. CAT

The female undergoes considerable psychological change with the onset of estrus. The first signs of impending heat are usually increased affection and tendency to roll and rub against objects. Many cat owners have mistaken this change in attitude for signs of illness. The rolling tendencies intensify and lordosis is exhibited during the first part of estrus until some cats will not walk, preferring to move with the elbows, chest, and abdomen on the ground and the pelvis elevated. A curious, low vocal sound is often emitted during estrus. A loud, sharp cry is emitted by the female following a certain intensity of coital stimulation. Following coitus, females usually roll spasmodically and groom their genitalia. Contrary to some previous reports, females allow coitus to be repeated within a few minutes with as many as seven intromissions having been observed for one female in one 2-hour test period (71).

The male cat often will make a chirping sound prior to mounting the female. To mount, the male grabs the back of the neck of the female. This is followed by paddling motions with the hind legs along the lateral abdominal wall of the female. The male's penis, which in a nonerect state points caudally, has reached full erection by this time, and is turned cranioventrally at 20 to 30° to the horizontal. The female elevates the vulvar area into a more horizontal position through arching of the back. This facilitates the entry of the penis into the vagina. The coital cry of the female is followed by a rapid dismounting of the male to avoid the female's aggressive afterreaction.

It is important that a male be well-adjusted to his surroundings before beginning a breeding program (17). It has been found that a male may require several days to a week to establish himself in a strange territory before he will actively engage in copulation. An estrous female cat will frequently be met with aggressive behavior by a nonestablished male (69).

#### IV. Reproductive Physiology of the Male Dog and Cat

##### A. THE MALE GENITALIA AND ACCESSORY GLANDS

Anatomical features of significance in coitus have been described. An added unique attribute of the feline penis is the presence of 100 to 200 cornified papillae on the corpus cavernosum glandis. These papillae, first observed at 6 to 7 months of age, appear to be androgen-dependent in that they diminish in size after castration. Their function in the stimulation of ovulation in the female has not been established (61).

The prostate is the only accessory gland found in the dog. It is normally situated on the anterior pelvic brim and completely encompasses the proximal urethra. It is divided into two closely apposed lobes. In the cat, the prostate is located about 2 to 3 cm caudal to the urinary bladder. In contrast to the dog, the prostate does not completely surround the urethra but is divided into two distinct lobes which are joined dorsally only, and lie on each side of the urethra.

The prostatic secretion in the dog is rich in sodium and chloride ions, but is low in citric acid (7, 98). The pH of canine prostatic fluid is  $6.8 \pm 0.33$  (95). Normal function of the prostate is androgen-dependent with testicular androgens transported to the prostate by the vein of the vas deferens (73).

In addition to a prostate, the cat has two bulbourethral glands that lie caudal to the prostate at the posterior rim of the pelvis. Other male accessory glands are absent in the cat as well as in the dog.

Meyer (68) has reported a positive correlation between the time of completion of deciduous dentition and the passage of testes through the inguinal canal in the German shorthair pointer with both events occurring between 30 and 35 days of age. The testes complete their descent into the scrotal fundus by the 63rd day.

The testes of the cat are descended at birth. The combined testicular weight increases from 20 mg at birth to 100 mg at 6 weeks. Seminiferous tubule mitotic activity is observed at 400 to 500 mg of weight, or at about 5 months of age. Spermatozoa appear in the ejaculatory fluid at 8 to 12 months of age (79).

## B. SPERMATOGENESIS

The absolute duration of the cycle of the seminiferous epithelium in the sexually mature beagle is 13.6 days (30). Testicular estrogens may regulate the release of interstitial cell-stimulating hormone (ICSH) in the dog via their effect on LHRH/FSHRH. As little as 0.025 mg of estradiol-17 $\beta$  administered 2.5 hours before an injection of LHRH/FSHRH successfully blocked ICSH release while testosterone, dihydrotestosterone (DHT), and progesterone failed to block the effect of the releasing hormone (56). The transport of DHT needed for spermatogenesis may occur by lymph flow as well as by the vascular route (41).

## C. SEMEN

Canine semen is ejaculated in three fractions. The first fraction, clear and sperm-free, is released before full erection is attained. The second fraction is ejaculated in conjunction with the intense ejaculatory reaction;

this is the sperm-bearing portion. The third fraction consists of clear fluid which is ejaculated while the animals are locked together. The time of collection of the first and second fractions, obtained manually in the presence of a teaser bitch, are similar to those obtained by the use of a uterine fistula during natural coitus (25).

It appears that relatively stable numbers of spermatozoa can be obtained if collection of semen occurs every other day in the dog. The percentage of morphologically normal spermatozoa and the libido of the male were not affected by an ejaculation frequency as often as twice daily, although there appeared to be reduced motility of sperm from the more frequent ejaculations when stored in extender at 5°C (8).

A pH of 7.0 to 8.5 is an optimal range for maintaining the motility of canine spermatozoa, although tolerance of pH ranges of 5 to 10 has been reported (95). While alkalinity is well-tolerated, spermatozoa become more susceptible to hypertonicity as the pH increases. The use of glucose in this situation has been beneficial. Diluents containing 0.02 M carbonate/bicarbonate buffer severely depress the motility of canine spermatozoa (95).

Dog semen has a very low fructose concentration which may be associated with the absence of vesicular glands.

Semen volume usually ranges from 0.01 to 0.12 ml in the cat, although 0.3 ml is occasionally obtained. The total sperm number per ejaculate is about  $57 \times 10^6$ . Sperm motility ranges from 60 to 90%. Abnormal sperm average about 10% and cytoplasmic droplets and club tails are the most prevalent types. The pH of cat semen is 7.4. Cat semen, like the dog semen, is low in fructose (86).

Semen can be collected from the cat three times weekly without decreasing volume or concentration of sperm. Daily collection causes a drop in volume and, at least initially, a depression in sperm concentration (86).

Cat spermatozoa require at least 2 hours in the uterus before fertilization *in vitro* can be accomplished (42). This could correspond to the capacitation time for the cat. Cat seminal plasma contains a reversible, partially heat-labile factor, which has an antifertility effect on rabbit spermatozoa (67). No decapacitation activity was found in the seminal plasma of dogs (23).

#### D. ARTIFICIAL INSEMINATION

Considerable progress has been made in recent years in the utilization of artificial insemination, including the storage of semen in the dog. The reader is referred to a complete review of the relative merits of procedures and equipment (83). Manual collections appear to be the most satisfactory

way to obtain semen (8, 65). Schutte and Bezuidenhout (77) found manual collection and the use of the artificial vagina equally effective. The artificial vagina appears cumbersome to use, especially in small breeds, and prolonged contact of semen with the warm, latex lining of the artificial vagina adversely affects motility (8). Electroejaculation produces a low volume of semen, often contaminated by urine, and may cause physical discomfort (34).

The dilution of the sperm-rich fraction of the ejaculate for short-term storage is most successful when doubly pasteurized skim milk is used as an extender. Semen diluted at 1:4 or 1:5 can be stored at 4° to 8°C with conception rates of 77% recorded for fresh semen and 53% for semen stored for 3 days (83).

Canine semen has been successfully frozen and stored for at least 19 months with an overall conception rate of 72%. The diluent that has been used is lactose (11%), glycerine (4%), and egg yolk (20%). A long equilibration period is not necessary prior to freezing when semen is pelleted directly on dry ice followed by storage in liquid nitrogen. The pellets are thawed rapidly in sodium citrate prior to insemination (84).

Artificial insemination in the cat is limited, at the present, to colonies where males can be trained to ejaculate into an artificial vagina. Insemination of 0.1 ml of semen diluted with saline to contain  $5 \times 10^6$  sperm/ml has resulted in a conception rate of 50% with one insemination and 75% following two inseminations 24 hours apart (86). Females must be in estrus and must be injected with ovulation-inducing hormones such as chorionic gonadotropin prior to insemination.

## V. Genetic Aspects

The normal diploid karyotype of the domestic dog (*Canis familiaris* L.) consists of 76 acrocentric or telecentric autosomes, a submetacentric X sex chromosome, and a minute metacentric Y sex chromosome ( $2n = 78$ ) (53). The karyotypes appear to be identical in all races of dogs. In the cat, the chromosomes are not as uniform. There are 32 metacentric or subtelecentric and 4 acrocentric or telecentric autosomes, a subtelecentric X and a subtelecentric Y for a total of 38 ( $2n$ ) (54). Buccal epithelial cells of both the dog and the cat show chromatin structures which are morphologically and histochemically (Feulgen-positive) comparable to the human sex chromatin (Barr body). These structures have been found in female dogs and cats with a frequency of 45.8 and 29.4%, respectively. The occurrence rate in male dogs and cats per 100 cells examined is 0.0017 and 0.038%, respectively (90).

Abnormal gonadal development, with subsequent abnormal differentiation and infertility, usually occurs in animals with abnormalities of sex chromosomal constitution or number, but can also occur when the sex chromosome constitution appears to be normal. This has been reported in cases of male pseudohermaphroditism of dogs in the presence of a 78,XX karyotype (20-44).

Chromosomal errors in the cat have been recognized through the phenotype tortoiseshell in males. Orange and black hair color genes are allelic on the X chromosome and males showing both colors must have two X chromosomes (plus a Y chromosome). Among the reported cases, some were found to have a 39,XXY constitution, but were more frequently chimeras with two or more populations of cells (38 XX/38,XY 38 XXY/39,XXY) (6). A case of a diploid/triploid (38 XX/57,XXY) chimera has been reported in a male tortoiseshell cat (16).

The ability of gray wolves and coyotes to interbreed with domestic dogs and produce fertile hybrids is well established (10). The possibility of fertile crossings between various species of jackals and the domestic dog is a matter of dispute (10), but successful matings have been reported between the dog (miniature poodle) and the Golden jackal (*Canis aureus*) as well as with the side striped jackal (*Canis adustus*) (48). Although all female offspring came into estrus twice per year, only a few fertile matings occurred.

Immunogenetic markers have been used successfully in determining paternity in the dog by positive identification or exclusion of the sire (93).

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# 20. Reproduction in Poultry

Janice M. Bahr and A. V. Nalbandov

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## I. Introduction

The reproductive physiology of the chicken is extremely fascinating and challenging. Unlike the extended estrous cycle of the mammal, the events of the ovulatory cycle of the chicken are compressed into a number of hours. The alternation of structures which occurs during the follicular and luteal phase of the mammalian cycle, are absent in the chicken. While the ovarian steroid and pituitary hormones are similar to those of the mammal, the signaling mechanism by which the ovarian steroid hormones control the hypothalamo-hypophyseal system in the chicken may be different from that of the mammalian system. This relationship which is well understood in the mammals still needs additional study in the chicken before its details become known. In this chapter we will try to elucidate some of these hormonal relationships which need to be answered and point out some of the problems which demand additional investigation and understanding.

## II. The Female Reproductive System

### A. THE OVARY

In the chicken, unlike the mammal, only the left ovary and oviduct are functional while the contralateral right ovary and oviduct usually remain as vestigial structures. It is now known that estrogen secreted by the left ovary during the posthatching period usually prevents development of the right ovary (38). The left ovary is located in the body cavity, ventral to the aorta, posterior to the vena cava, and cranial to the kidney. The avian ovary differs morphologically from the mammalian ovary in that it has two major lobes within which are a large number of follicles carried on follicular stalks. A rich extensive vascular system supplies the ovary and its numerous follicles. The neural components of the ovary have been examined most thoroughly by Gilbert (22-24), Freedman (18), and Dahl (9). The ovary is well-innervated with a neural supply derived from an extensive network of ganglia, nerve cells, and nerves lying adjacent to and within the ovarian stalk. Both cholinergic and adrenergic nerves are associated with the blood vessels and smooth muscles of the medulla and cortex of the ovary. Some nerves extend to the developing and mature follicles.

In the immature ovary, there are thousands of oocytes. At the time of sexual maturity (18-20 weeks of age) 4 to 6 of these oocytes increase in diameter and the follicular hierarchy begins to be established. During the subsequent ovulatory cycles, only the largest follicles will ovulate followed on successive days by the second, third, and fourth largest ones, each of which enlarges to assume the size of its ovulated predecessor. It is not known what controls this follicular hierarchy or how and why certain follicles become part of the hierarchy while others do not but may join it in subsequent cycles. It has been postulated that the size relation of follicles may be determined by the vascular system which makes precursors (such as hormones and lipovitellin) more available to some follicles than to others.

The follicle consists of an oocyte and the surrounding six layers, namely, the vitelline membrane and zona radiata (innermost layer), perivitelline layer and granulosa membrane, theca interna, theca externa, connective tissue, and epithelium. Nerves and blood vessels penetrate the theca while the granulosa lacks both neural and vascular components (9, 22). Using electron microscopy, Dahl (9) has identified afferent terminals of both sympathetic (adrenergic) and parasympathetic (cholinergic) nerves in membranous contact with "steroid-producing" cells. As the follicle increases in size, the stigma becomes visible on the follicle. The stigma, a

specialized region of the follicle where the split occurs at ovulation, is a pale band approximately 2–3 mm wide and is supplied with very few small veins and arteries (49). During the development of the oocyte, yolk is laid down in three phases. The first yolk deposited is a neutral fat; later, yolk proteins are added. Approximately 7–11 days before ovulation, there is a very rapid accumulation of yellow yolk (25).

For a more comprehensive review of the avian ovary and oviduct see Gilbert (25).

## B. THE OVIDUCT

### 1. Structure

The oviduct, a long tortuous tube, extends from the ovary to the cloaca. The oviduct consists of five segments each having a separate function (Fig. 1). The segments are the infundibulum, magnum, isthmus, uterus, and vagina. The ovum spends varying amounts of time in each segment of

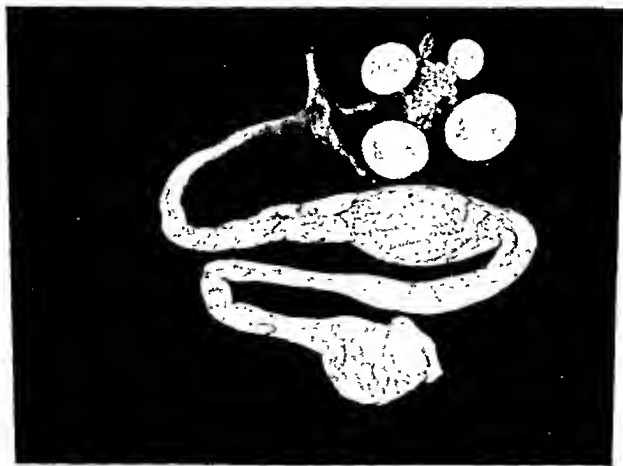


FIG. 1. The ovary and female reproductive tract of the chicken. Note the hierarchy of follicles, the ruptured follicle, and the numerous small follicles.

the oviduct, i.e., 0.25–0.5 hours in the infundibulum, 2.0–3.0 hours in the magnum, 1.25 hours in the isthmus, and 18–20 hours in the uterus (76). The oviduct has a rich nerve supply from both sympathetic and parasympathetic divisions with the uterus and utero-vaginal junction being most densely innervated.

The infundibulum, located at the anterior end of the oviduct, has a funnel shaped opening. At the time of ovulation, the infundibulum becomes very active as it tries to engulf the ovum. These wavelike movements are caused by vascular engorgement and muscular contractions of the infundibulum which are probably under neural and hormonal control. Whenever these mechanisms fail, the freshly ovulated ovum is deposited in the abdominal cavity (internal laying) and is gradually reabsorbed. Internal laying is more frequent during certain seasons than others (78). The fertilization of the egg and secretion of the perivitelline membrane, two other reproductive functions, occur in the oviduct (3).

The magnum, the longest part of the oviduct, is very conspicuous because of its dull white color. The thick wall of the magnum contains glandular tissue which secretes copious amounts of albumin as the ovum moves through the magnum in a rotating motion.

The isthmus, the next section of the hen's oviduct, secretes the shell membranes around the ovum which has been coated with albumin. One of the principal constituents of the shell membranes is ovokeratin (60).

The uterus or shell gland, as it is sometimes called, is a large expanded part of the oviduct. Here the egg is detained approximately 20 hours or more as the shell is laid down. Porphyrins, which are secreted by the uterine epithelium, cause a distinct egg coloring in some avian species.

The vagina is a short S-shaped tube which probably has no role in the formation of an egg. Sperm either deposited by natural mating or artificial insemination are stored in the vaginal glands which are short, simple tubules near the utero-vaginal junction.

## 2. Secretion of Albumin and Shell Formation

For extensive reviews of the chemical nature and biological synthesis of egg albumin, see Baker (4) and Feeney and Allison (11).

Egg albumin contains numerous proteins. Using starch gel electrophoresis, Lush (44) identified nineteen major components of egg whites. Some of these proteins possess bacteriocidal activity. The biological function of the other proteins is still obscure. Feeney (11) has suggested that some proteins are found in the albumin to provide bulk material and to ensure correct amino acid milieu for embryonic development. Ovalbumin, the most abundant protein in egg white (54%) contains all essential

amino acids. Other principal proteins are ovotransferrin (13%) which binds polyvalent metals, ovomucoid (11%), an inhibitor of proteases, and lysozyme (4%), an enzyme.

While yolk proteins are formed at another site in the body and subsequently transported to the yolk, albumin is synthesized in the oviducal tissue (55). Though the oviduct may store albumin for 2 days, approximately 45% of the albumin can be rapidly produced as the egg passes through the magnum (16, 69).

Albumin formation is under hormonal control. According to Brown (5), testosterone acts synergistically with estrogen to promote oviducal growth while progesterone is antagonistic to the action of estrogen (56). The actual mechanism of the secretion of albumin is still questionable despite the fact that this problem has been investigated for many years. Gilbert (25) suggests three different possibilities for the regulation of albumin secretion: (a) direct mechanical stimulation by the ovum as it passes through the oviduct or the presence of a chemical substance diffusing from the yolk; (b) a humoral agent; and (c) a neural coordinating mechanism. There is considerable evidence for a direct mechanical stimulation of the oviduct by the ovum because albumin can be secreted around foreign objects placed in the oviduct. Without doubt, the control of albumin secretion needs further study.

In the formation of a shell, two fibrous membranes are first laid down by the isthmus. Then the outer shell which consists almost totally of calcium carbonate is secreted by the uterus or shell gland. At the same time there is watery secretion by the shell gland which "plumps" the egg. During shell formation,  $\text{Ca}^{2+}$  and other inorganic components of the shell are actively removed from the blood (63). The formation of a shell and also an egg, which is almost a daily occurrence during the ovulatory cycle, places tremendous metabolic demands on the hen.

At this time, it would be informative to point out that many of the activities involved in egg formation are under hormonal control, specifically the ovarian steroid hormones. Estrogen has several highly important functions in the metabolic economy of the laying hen. It plays a vital role in  $\text{Ca}^{2+}$  metabolism in that it is responsible for the transportation of ingested  $\text{CaCO}_3$  from the gut to the bone where it is deposited. When laying commences, about 5 gm of  $\text{CaCO}_3$  are required daily for the building of the shell. This Ca is mobilized by estrogen, in part, directly from the gut and, in part (by the action of osteoclasts), from the bone. Equally important is the action of estrogen directly on the liver (and to some extent perhaps on the fat depots), where the lipoproteins essential for yolk formation are mobilized, transported through the peripheral circulation, and enter the growing follicles. Since the follicle is one of the fastest growing biological

structures (it grows from a weight of micrograms to a weight of about 24 gm in 8 days), the amount of lipoprotein deposited in the fastest growing follicle may reach 2 gm or more in 24 hours. The amount of lipoprotein mobilized by estrogen and present in the blood makes the laying hen have the highest level of hyperlipemia known in any animal.

Estrogen is also essential for the building of uterine glands in the growing pullet. These estrogen-built and maintained secretory glands, when acted upon by either progesterone or testosterone (it is still not clear which!), cause the magnum to secrete ovalbumin.

### 3. Oviposition

Generally, 25–26 hours after ovulation, the egg is laid. The hen displays a distinct behavior, i.e., nesting, changes in respiration, and muscular contractions. Very likely oviposition is under both neural and hormonal control. The time of oviposition may be determined by ovulation via changes in the ruptured follicle (26). The postovulatory (ruptured) follicle is necessary for oviposition because its removal delays oviposition (26). Day (personal communication) has observed significantly elevated levels of the prostaglandin,  $F_{2\alpha}$ , in the ruptured follicle at the time of oviposition. This preliminary evidence suggests that prostaglandin  $F_{2\alpha}$  in the ruptured follicle may play a regulatory role in oviposition.

### C. THE OVULATORY AND OVIPOSITORY CYCLE

The hen lays eggs sequentially followed by one or more pause days. A clutch consists of the number of eggs laid on consecutive days in an *uninterrupted series*. Clutch lengths are generally of 6 days but range from 2 to 8 days and in some hens may be 360 or more days. An ovulatory cycle extends from the oviposition of the first egg of a clutch until oviposition of the first egg of the next clutch. Generally the laying cycle of the hen is governed by the lighting schedule. If the hen is given 12–14 hours of light per day, the first egg of the clutch is usually laid early in the morning. As a rule, ovulation occurs shortly after oviposition (5–60 minutes), and approximately 26 hours are required before the newly ovulated ovum acquires its integuments and is ready to be laid as an egg. Because ovulation does not occur until after oviposition and because the completion of the formation of the egg requires at least 24 hours, each succeeding egg in the clutch is laid later than the first eggs of the clutch; the last egg of the clutch is laid early in the afternoon. The hens with the longest clutches ovulate



very shortly after oviposition and take less time (some only 24 hours) to complete the egg. If hens are placed under continuous light the laying schedule outlined above is interrupted and egg laying will occur at random throughout the 24-hour period. Apparently light-dark sequences are normally used as environmental cues for the release of the hormones involved in the control of ovulation. Interestingly, other environmental cues may be substituted. Thus, in hens placed on constant light, ovulations will continue to occur predominantly during the "morning" hours provided the daily activities, such as feeding and cleaning, continue to be performed during the "morning" hours. The interval between ovipositions is usually greater than 24 hours and the interval minus 24 hours is called "lag." The lag is greater between the first and second egg in the clutch, then decreases during the middle of the cycle and increases again toward the end of the cycle. The ovulatory cycle is hormonally controlled by the subtle interaction of the gonadotropins and the ovarian steroid hormones. This complex phenomenon will be discussed in the next section.

#### D. HORMONAL CONTROL OF THE OVULATORY CYCLE

##### 1. Theory of Ovulation

Several theories have been proposed to explain ovulation in the hen. An acceptable theory of ovulation must explain not only the interrelationships of the gonadotropins and ovarian steroid hormones but also why the hen will ovulate consecutively for several days and then skip a day. One of the more interesting theories of ovulation has been proposed by Fraps (13). He suggested that follicular growth and maturation in the hen depends on a constant output of FSH by the pituitary with basal levels of LH. At specific times during the ovulatory cycle there is an increased release of LH which causes ovulation. An "excitation hormone" acts via the neural pathway (hypothalamus) to trigger the LH release. The threshold for the action of the "excitation hormone" undergoes diurnal fluctuations. If the level of the excitation hormone reaches the threshold level necessary for the LH release, the gonadotropin is released and ovulation follows. Since successive follicles mature at intervals of 24 hours plus a lag (at a later time each day), the increase in the excitation hormone would follow a similar time course. As a result the neural components would be stimulated at a progressively later time each day until eventually the peak level of the excitation hormone would occur at a time when the neural threshold would also be elevated. Because the given concentration of the excitation hormone was not sufficiently high to trigger the LH release, no

ovulation would occur on that day and there would be a pause in the ovulatory cycle. LH would be stimulated again the next day when the neural threshold for LH had decreased and ovulation would again resume. Though Fraps did not identify the excitation hormone, it is generally assumed to be progesterone. It should be remembered that this theory was suggested before actual measurements of both the gonadotropins and the ovarian steroids were technically feasible. As a result, this theory does require some modification and additions before it can adequately describe ovulation. While keeping this theory in mind, let us examine some of the recent studies in which measurements of hormones were reported.

Because of the precise and sensitive technique of the radioimmunoassay (RIA), it is now possible to measure the gonadotropins and the ovarian steroids simultaneously in the same hen. There have been many efforts to test Frap's theory and to correctly identify the "excitation hormone."

It is very challenging to disentangle the hypothalamo-hypophyseal-ovarian relationships in the hen because all these changes are compressed into a 24- to 26-hour ovulatory cycle unlike the extended mammalian estrous cycle. Furthermore, it appears that the steroid hormones from the maturing follicles are also part of the complicated picture. At the present time we do not know whether the entire hierarchy of the different sized follicles or only the largest follicle gives the signal necessary for the release of the hormones which cause ovulation.

## 2 Induction of Ovulation

In discussing the hormones involved in ovulation we must consider two *modi operandi*. First, we know that hypophyseal hormones (LH and FSH) are the immediate cause of follicular rupture. Second, it is important to ask what is responsible for the timing of the release of the hypophyseal ovulation-inducing hormone (OIH). In the latter category all three ovarian steroids, estrogen, progesterone, and testosterone will have to be considered. It should be noted that in mammals ovarian estrogen (estradiol-17 $\beta$ ) has been conclusively demonstrated to be able to release ovulatory doses of hypophyseal LH (and possibly FSH). In chickens, Fraps (12) found that an injection of 1 mg of progesterone caused 95% of 19 hens to ovulate prematurely. A similar dose of testosterone ovulated 41% of 32 hens while 1 mg of estradiol-17 $\alpha$  had no effect on ovulation. These earlier data have been confirmed by Lague *et al.* (39) who reported that a dose of 500 to 1000  $\mu$ g of P<sub>4</sub> induced premature ovulation in 100% of the hens. When both estradiol-17 $\beta$  and progesterone were injected, no facilitatory or inhibitory effect of estradiol-17 $\beta$  was observed on the spontaneous or progesterone-induced ovulations. In another study, Fraps and Dury (14)

reported that an injection of either LH or PMSG induced premature ovulation in hens. Later Opel and Nalbandov (57) reported that ovine LH could cause ovulations in hypophysectomized hens ( Figs. 2 and 3). Similar results were obtained by Tanaka *et al.* (72) who injected ovine LH and caused ovulation in hens whose hypothalami were blocked with phenobarbital sodium and thus did not cause release of endogenous LH. When LH was omitted no ovulations occurred. These results indicate, first, that the ovarian steroid which appears to be necessary for the release of the ovulation-inducing hormone(s) is progesterone and, second, that LH and FSH(?) are the ovulation-inducing hormones. These preliminary studies do not elucidate whether progesterone is acting on the hypothalamus or directly on the pituitary to cause ovulation.

### 3. Measurement of Ovarian Steroids

It is now accepted that ovarian steroids have an important role in the overall metabolism in the hen. Reference to some of these effects was made earlier in this chapter (see Section II,B,2). For a more detailed discussion of this topic, we refer you to extensive reviews by Gilbert (25). Marlow and Richert (46), in 1940, reported that estrogen is secreted by the hen's ovary. Since that time there have been numerous reports identifying both estrone and estradiol-17 $\beta$  in the hen's plasma (43, 53). More recently the estrogens have been quantitated during the entire ovulatory cycle (see below).

Another important ovarian hormone is progestin which was first identified in avian blood by Fraps *et al.*, in 1948 (15). Similar findings have been reported by O'Malley *et al.* (54), Layne *et al.* (42), Lytle and Lorenz (45), Furr (19), Arcos and Opel (1), and others. Surely, to many investigators, the presence of relatively high progesterone levels in the hen's blood were perplexing because in contradistinction to mammals, the laying hen possesses no structure analogous to the corpus luteum which is a rich source of progesterone.

The third ovarian hormone present in rather copious amounts in the hen is testosterone. Witschi and Fugo (77) suggested that the ovary secretes androgens which were responsible for the bright appearance of the comb. In 1966, Woods and Domm (79) identified androgens in ovarian tissue by using the fluorescent antibody technique. Using gas chromatography, O'Malley (54) and Furr and Thomas (21), measured testosterone levels ranging from 20 to 120 ng/100 ml in avian blood. Very recently, Shahabi *et al.* (67) measured testosterone, estrogen, and progestin levels in both the plasma and follicles of hens during the entire ovulatory cycle. These results will be presented and discussed in a later section.

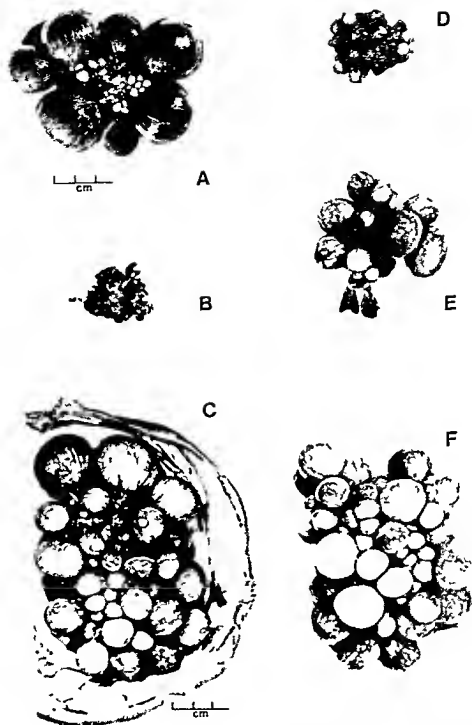


FIG. 2 (A) Ovary of normal chicken. Note hierarchical arrangement of follicle sizes. At the bottom is the empty follicle sac of the most recently ovulated follicle. If a normal hen is injected with an LH-containing hormone, only one, the largest follicle, will ovulate prematurely. (B) Totally degenerated ovary of a hen 48 hours after hypophysectomy. (C) Ovary and enlarged oviduct of normal hen injected with PMSG. Note the overstimulation and the fact the hierarchy has been abolished. If

#### 4. Measurement of LH and FSH

The fact that injections of LH could cause premature ovulation makes it important to measure LH during the ovulatory cycle and correlate changes in LH concentrations with ovulation. Nelson *et al.* (52) determined LH levels in the pituitary and plasma during the ovulatory cycle of laying hens by using the ovarian ascorbic acid depletion (OAAD) bioassay. They reported LH peaks at 8, 13, and 21 hours before ovulation. These results were confirmed by Bullock (6), and Bullock and Nalbandov (7). The latter further concluded that whenever the peaks of LH were absent, no ovulation occurred on that day and thus there was a pause in egg laying. Tanaka and Yoshioka (73), also measuring pituitary LH by bioassay, found peak levels only at 20 and 8 hours before ovulation of the second egg in the clutch. More recently, using a radioimmunoassay with an antibody specific to avian LH, Furr *et al.* (20) detected only one LH peak (3.09 ng/ml) 4-7 hours before ovulation. Using the same system as Furr *et al.* (20), Shodono (68) also identified one LH peak of 4.36 ng/ml at 5 to 4 hours before ovulation. Besides measurements of LH, Tanaka *et al.* (71) measured the gonadotropin-releasing activity of the stalk-median eminence during the cycle. They reported LHRH peaks at 21 and 8-11 hours before ovulation, e.g., at two of the times when LH peaks were found to occur by OAAD. At this time there is no acceptable explanation for the discrepancy in results obtained from the bioassay and radioimmunoassay. We will return to this problem in Section II,D,5, when we discuss synthesis of steroids by follicles. Since major differences have been found between these two systems in other species (58), it is possible that the results obtained from bioassay and radioimmunoassay differ because they may be detecting various species of LH molecules. One must bear in mind that while the bioassay measures the biological site, the RIA detects the immunological sites. The data of Nelson *et al.* (52) and Bullock (6) have been criticized because Jackson and Nalbandov (33) found that chicken pituitary glands contain arginine vasotocin which is known to act like LH (e.g., cause ascorbic acid depletion) in the OAAD assay system. However, it should be pointed out that the LH peaks discovered in the OAAD assay

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such hens are injected with LH (IV), all the largest follicles can be made to ovulate. (D) Minimal stimulation of the ovary of hypophysectomized hens is possible with mammalian gonadotropins. (E) With whole chicken pituitary extracts restoration of the ovary is possible in hypophysectomized hens. A semblance of the normal follicular hierarchy is achieved. (F) Just as in the intact hen (C), the ovary of a hypophysectomized hen can be overstimulated with either PMSG or hypophyseal FSH and LH (mammalian) but the hierarchy of size is not evident. (All magnifications are the same:  $\times \frac{1}{2}$ .) From Opel and Nalbandov (57).

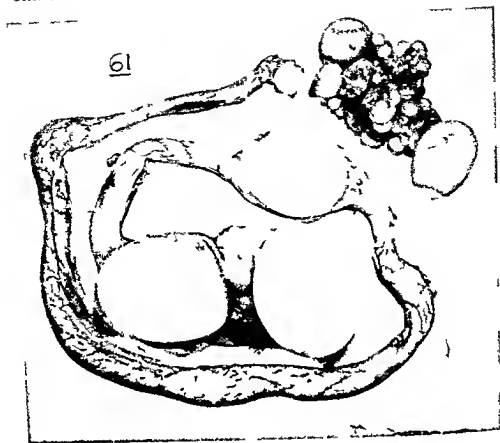


FIG 3 Multiple ovulations can be induced in hypophysectomized hens by the injection of LH containing gonadotropins (Note three eggs in the duct system one of which is ready to be laid) In contrast in intact hens only the largest follicle can be induced to ovulate Superovulation is the rule in hypophysectomized hens or in hens whose ovaries have been overstimulated with exogenous gonadotropins From Opel and Nalbandov (57)

occur in the plasma which bathes the growing follicle and it appears highly improbable that sufficiently high levels of vasotocin would be present in plasma to be mistaken for LH. Despite the differences in some of the results, both the OAAD and the RIA agree on an LH peak 4-7 hours before ovulation.

Since LH appeared to be the ovulation inducing hormone, little attention has been given to FSH. However, studies by Kao and Nalbandov (35) and Shahabi *et al* (66), suggest that both FSH and LH may be required for ovulation. Kao and Nalbandov found that injections of the  $\alpha$  adrenergic blocking agent, Dibenzylamine, directly into chicken follicles blocked the subsequent ovulation of that follicle. This blockade by the drug could be overcome only by the injection of a mixture of FSH and LH. In another study, Shahabi *et al* (66) found that an injection of ovine LH 12 hours

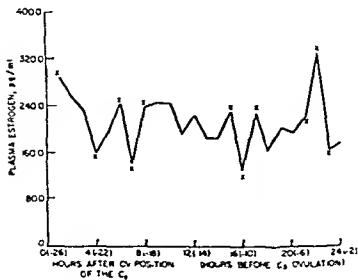
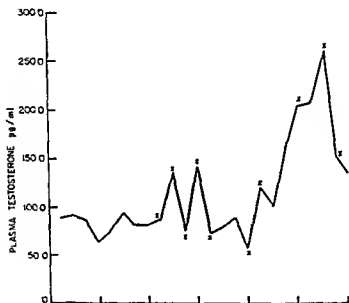
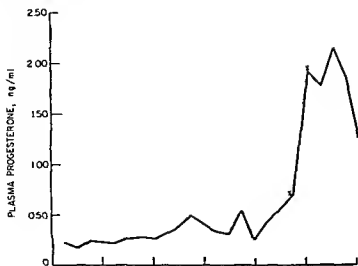
before ovulation of  $C_3$  (the third egg in the clutch) induced the identical steroid pattern seen 5 hours before ovulation with the exception of estrogen concentrations. It is possible that both LH and FSH are needed to elevate estrogen levels in the follicle and plasma.

FSH levels during the ovulatory cycle of the hen have been reported by Kamiyoshi and Tanaka (34) and Imai and Nalbandov (31), using the modified Steelman-Pohley bioassay. Kamiyoshi and Tanaka (34) found that pituitary FSH content fluctuated during the ovulatory cycle of the hen with a peak release of FSH from the pituitary approximately 11 hours before ovulation. On the other hand, Imai and Nalbandov (31) reported two peaks in plasma with the first peak occurring 1 hour after oviposition of  $C_1$  and the second peak 11 hours before ovulation of  $C_3$ . They measured FSH peaks in the pituitary at 4.5 hours after oviposition of  $C_1$  and 14 and 5 hours before ovulation of  $C_3$ . Imai and Nalbandov suggested that the first peak in the plasma was necessary for the rapid growth of the next follicle ( $C_2$ ) while the second FSH plasma peak, along with the LH peak, was needed for follicular maturation and ovulation. If the avian ovulatory cycle follows a model similar to that of the mammalian, then one would expect that both gonadotropins are required for subtle interactions and synergistic responses.

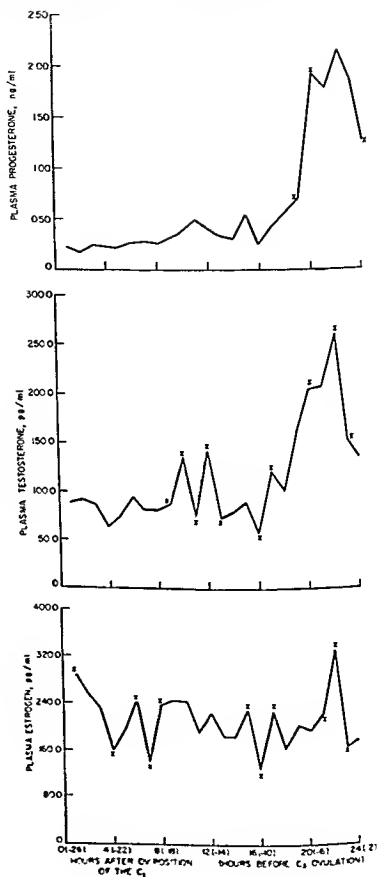
## 5. Interrelations of Gonadotropins and Ovarian Steroids during the Ovulatory Cycle

In mammals, when a mature follicle ruptures it is transformed into a corpus luteum which predominates during a part of the cycle (luteal phase). Later a new crop of follicles begins to mature and, concurrently with the decline of the corpus luteum, follicles become predominant (follicular phase). During the luteal phase, the most prominent ovarian hormone is progesterone while during the follicular phase it is estrogen. Thus, the hormones impinging on the hypothalamo-hypophyseal system alternate and with them the signals registered by this system differ. It is now well established in mammals that the rise in follicular estrogen during the later stages of the follicular phase is responsible for the release of hypothalamic gonadotropin releasing hormone (GnRH) which, in turn, leads to the release of hypophyseal FSH and LH which cause ovulation.

The chicken has only a continuous follicular phase and in it ovulation occurs daily (within the limitations of the clutch). Since follicular growth is an ongoing process, the question arises whether follicular steroid synthesis is also a continuous process. If not, how is it regulated in detail since it has been established that chicken gonads totally depend on hypophyseal tropic function for normal gametogenic and steroidogenic function (57).







Another question that will be asked concerns the signal which tells the chicken hypothalamus that the largest follicle is ready to ovulate and that the ovulation-inducing hormone should be released. Also of interest is the question of which of the three ovarian steroids serves as the signal. Finally, an interesting and yet unsolved problem concerns the question whether the steroids only from the largest follicle do the signaling or whether *all* large follicles participate. Why this is of interest will be discussed shortly.

In an effort to demonstrate and understand the relationship between the gonadotropins and ovarian steroids there are currently several reports in the literature in which LH and/or ovarian steroids were quantitated during the entire ovulatory cycle of the hen. Shahabi *et al* (67) measured estrogen, testosterone, and progesterone in plasma and from the three largest follicles during each hour of the ovulatory cycle in the same hens to determine the rates of synthesis and release of the ovarian steroids by the follicles. Despite the difficulty of comparing results from various reports because of the differences in sampling time, some general conclusions can be drawn.

Shodono *et al* (68), measuring estradiol-17 $\beta$ , progesterone, and LH in the plasma of hens during the ovulatory cycle found elevated estradiol-17 $\beta$  levels at 24 hours and a significant peak at 7 to 5 hours before ovulation. Similarly, Peterson and Common (59) reported peaks of estradiol-17 $\beta$  at 18 to 22 and 2 to 6 hours before ovulation. On the other hand, Senior and Cunningham (65) and Senior (64) detected only one peak in estradiol-17 $\beta$  and estrone approximately 6 hours before ovulation. These authors also demonstrated that the day on which no ovulation occurs, there is no estradiol-17 $\beta$  peak in the plasma. In contrast to these studies, Shahabi *et al* (67) (Fig. 4) found three significant peaks of estrogen in the plasma during the ovulatory cycle. These peaks occurred at 16 to 17, 8, and 4 hours before ovulation. Some of the discrepancy in these results may reflect the intervals between samples, the accuracy with which ovulation was timed, and whether total estrogens or a specific estrogen was measured. There is complete agreement in the literature regarding progesterone peaks during the ovulatory cycle. Shodono *et al* (68), Furr *et al* (20), Peterson and Common (59), Kappauf and van Tienhoven (36), and Shahabi *et al* (67) all reported one major plasma progesterone peak (2.0 to 6.0 ng/ml) approximately 4-6 hours before ovulation.

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FIG. 4. Plasma levels of progesterone, testosterone, and estrogen throughout ovulatory cycle. Note that with the possible exception of estrogen the plasma peaks of testosterone do not correspond to the follicle peaks until just before ovulation when all three steroids show highly significant peaks in the plasma. From Shahabi *et al* (67).

Even though testosterone had been identified in the hen's plasma many years ago and had been demonstrated to be necessary for comb formation and maintenance, its measurement and physiological significance have been overlooked by most investigators. Shahabi *et al.* (67) determined a testosterone concentration in plasma each hour from the time of ovulation to time of oviposition of the C<sub>3</sub> egg. They found significant testosterone peaks in the plasma at about 14 to 16 hours, and at about 4 to 6 hours before ovulation of C<sub>3</sub>.

In still other reports, Shodono *et al.* (68), Furr *et al.* (20), and Senior and Cunningham (65) measured LH and either estradiol-17 $\beta$  or progesterone simultaneously to determine which ovarian hormone is the excitation hormone. Since all three ovarian steroids peak simultaneously approximately 6 hours before ovulation, it is impossible to ascertain from these studies which ovarian steroid is responsible for the LH peak which follows shortly thereafter. Furthermore, there are no LH, estradiol-17 $\beta$ , or progesterone plasma peaks on the day in which no ovulation occurs. However, if one wants to assume that the hypothalamo-hypophyseal-ovarian interrelationships in birds is similar to that which exists in the mammal, then estradiol-17 $\beta$  would be the ovarian hormone which would trigger the LH release. In rats (62), ewes (27, 61), monkeys (37), and many other mammals, there is adequate evidence to conclude that estradiol-17 $\beta$  stimulates the ovulatory surge of LH. Even though a preovulatory peak of progesterone does occur in the rat, Ferin *et al.* (17) have shown that neutralization of this progesterone peak with antibodies does not interfere with ovulation. Moreover, there is no preovulatory progesterone peak in the cow (70), sow (74), monkey (51), and man. It is very possible that because of the uniqueness of the ovulatory cycle in the chicken, that not one ovarian steroid, but all three steroids (estrogen, testosterone, and progesterone) trigger the preovulatory LH peak since all three ovarian steroids peak in synchrony approximately 6 hours before ovulation (67).

In the above discussion we are presenting evidence to substantiate the idea that one or more ovarian steroids trigger the preovulatory surge of LH as has been suggested from a number of current papers. Yet, we are in a dilemma to explain why a systemic injection of LH 12 hours before ovulation can elevate both the plasma and follicular steroids, with the exception of plasma estrogen, to identical levels observed for the steroids 6 hours before ovulation (66). In other words, despite the strong evidence that a steroid(s) acts as the "excitation hormone" to trigger the preovulatory LH surge, as Fraps has hypothesized, there is still some question if the synchronous peaks of steroids 6 hours before ovulation or the significant plasma peaks of testosterone and estrogen 11-9 hours before ovulation are responsible for the preovulatory surge of LH.

An examination of steroid concentrations in the three largest follicles will complicate the story even more. A different steroid profile is seen for each follicle (67). Totally contrary to expectation is the finding that the concentrations of all three steroids (estrogen, testosterone, and progesterone) were lowest in the largest follicle, the one destined to ovulate next (Fig. 5). The testosterone and estrogen concentrations dropped precipitously immediately after the ovulation of the previous follicle. On the other hand, progesterone concentration in all three follicles (only one of which ovulated!) was low and remained low until approximately 6 hours before the next ovulation when the progesterone concentration rose very dramatically in the largest follicle. As can be seen in Fig. 5, testosterone and estrogen concentrations peak in the three largest follicles following oviposition ( $\sim 22$  to 18 hours before ovulation) and immediately preceding ovulation. What is of real interest is the observation that while progesterone is present in the greatest concentration in the largest follicle, testosterone and estrogen are present in the greatest concentration in the second and third follicles, respectively. How and why each follicle responds independently to tropic substances during the ovulatory period is not known.

The inverse relation between steroid concentration and follicle size may be due to a more rapid rate of steroid synthesis by the smaller follicle but equally plausible is the possibility that the lower steroid concentration and content of the follicle destined to ovulate next may be due to a more rapid release rate of steroids than that of which smaller follicles are capable. Thus, the enigma of whether the whole ovary participates in signaling the hypothalamo-hypophyseal axis that GnRH and, hence, the ovulation-inducing hormone should be released, or whether the largest follicle destined to ovulate next determines alone its destiny, remains unknown. Furthermore, which one, two, or three of the ovarian steroids do the signaling remains to be solved and leaves a fruitful but difficult field of research which challenges the imagination of the scientists interested in such problems.

## E. THE MOLTING HEN

Generally once a year, for approximately a month, many hens (especially of some breeds) stop laying eggs and molt. The cause of this interesting phenomenon has intrigued many reproductive physiologists. Evidence accumulated during the past 30 years indicates that molting is under endocrine control. In 1961, Nakajo and Imai (50) reported that the total gonadotropic content of the anterior pituitary was greater in the nonlaying than in the laying hen. The lowest pituitary content of gonadotropins was

found in the broody hen. Similarly, higher plasma gonadotropin levels were measured in the nonlaying hen than in the laying hen (30, 2).

Because the ovary also regresses in molting hens, it was not evident from these earlier studies whether the high pituitary gonadotropin levels and/or ovarian insensitivity to gonadotropins was the cause of molting. Imai *et al.* (32) designed an experiment in which they measured both plasma and pituitary FSH and LH levels and serum vitellin, which is a valid index of ovarian activity. According to the OAAD and modified Steelman assay, the pituitary FSH content of the molting hen ( $6.6 \mu\text{g/pituitary}$ ) was approximately twice that of the laying hen ( $3.9 \mu\text{g/pituitary}$ ). In contrast, the content of LH in the pituitary of the molting and laying hens ( $0.7 \mu\text{g/pituitary}$ ) was very similar ( $0.9 \mu\text{g/pituitary}$ ). Total gonadotropin activity of the pituitary increased as molting progressed.

Serum vitellin levels normally decrease within 5 days after the cessation of laying and increase again approximately 8–12 days before the first egg is laid. Injection of molting hens with estrogen caused significant

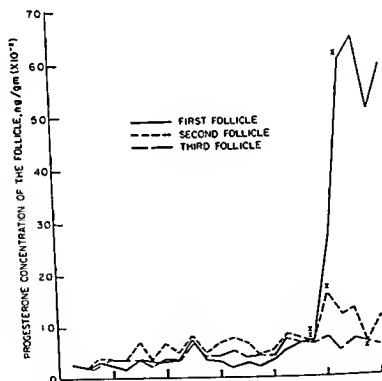
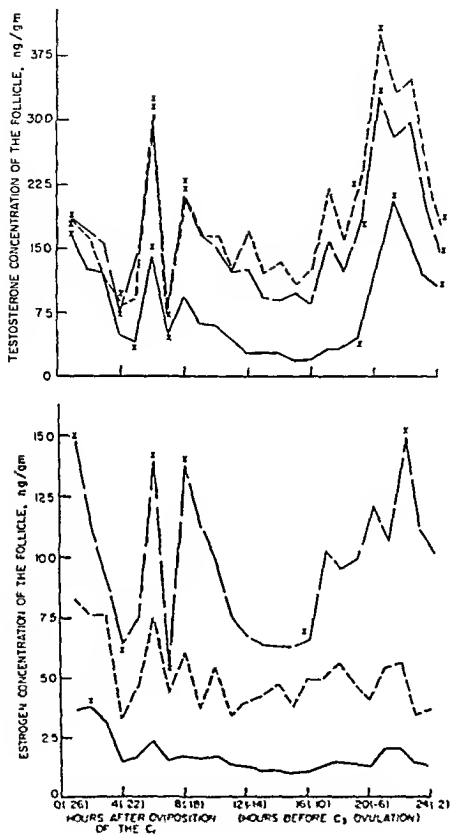


FIG. 5. Profile of steroid hormones in the follicle walls of laying hens throughout ovulatory cycle. Note that the two smaller follicles have a higher concentration of testosterone and of estrogen than does the follicle destined to ovulate next. Also note that both testosterone and estrogen show significant peaks about 18 to 20 hours prior to the next ovulation indicating increased rates of synthesis of these steroids. Note that all three steroid hormones peak in unison about 6 hours prior to the next ovulation (\*indicated significance at  $p = 0.01$ ). From Shahabi *et al.* (67).

FIG. 5. *Continued.*

increases in serum vitellin which suggests that if the liver is stimulated by estrogen, it can produce yolk precursors. Treatment of molting hens with PMSG caused the formation of yellow follicles and increased serum vitellin levels. Results from this study suggest that the cessation of egg laying

found in the broody hen. Similarly, higher plasma gonadotropin levels were measured in the nonlaying hen than in the laying hen (30, 2).

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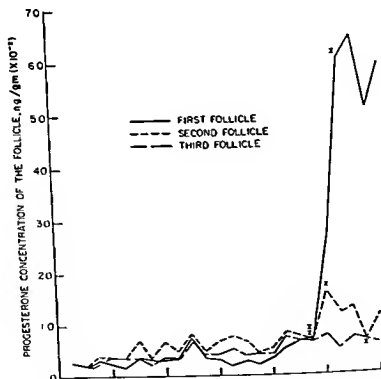
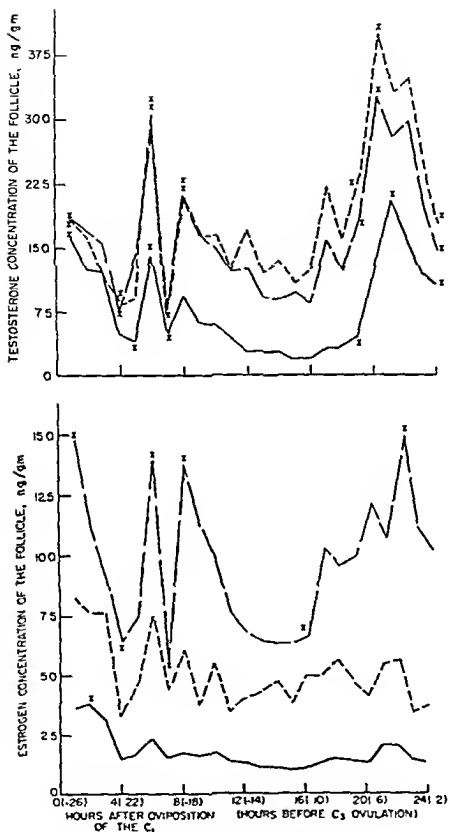


FIG. 5. Profile of steroid hormones in the follicle walls of laying hens throughout ovulatory cycle. Note that the two smaller follicles have a higher concentration of testosterone and of estrogen than does the follicle destined to ovulate next. Also note that both testosterone and estrogen show significant peaks about 18 to 20 hours prior to the next ovulation indicating increased rates of synthesis of these steroids. Note that all three steroid hormones peak in unison about 6 hours prior to the next ovulation (\*indicated significance at  $p = 0.01$ ). From Shahabi *et al.* (67).

FIG. 5. *Continued.*

increases in serum vitellin which suggests that if the liver is stimulated by estrogen, it can produce yolk precursors. Treatment of molting hens with PMSG caused the formation of yellow follicles and increased serum vitellin levels. Results from this study suggest that the cessation of egg laying



during molting is not the result of a change in the sensitivity of the ovary and liver to gonadotropins and estrogen or inadequate gonadotropin levels, but rather an unbalanced secretion of gonadotropins due to elevated FSH levels.

### III. The Male Reproductive System

#### A. ANATOMY OF THE REPRODUCTIVE TRACT

The structure and location of the avian male reproductive tract is significantly different from the tract of most mammals. Unlike the scrotal mammals, the paired testes are attached to the dorsal wall at the anterior end of the kidney. The accessory reproductive glands, i.e., prostate, seminal vesicles, which are found in most mammals, are absent in the cockerel. Furthermore, the pampiniform plexus of blood vessels which is necessary for temperature regulation of the mammalian testis is also lacking in the avians (75). In contrast, the avian testes function at a body temperature of 43°C.

Because the testes lack connective tissue septa, the tubules are not divided into distinct lobules (40). The seminiferous tubules, having a large diameter and high fluid content, are surrounded by blood vessels and Leydig cells. Only a small epididymis, attached to the dorsal surface of the testis, is present. The vas deferens, a long extensive convoluted tube, runs posteriorly along the medioventral surface of the kidney, parallel with the ureters, and terminates in the cloacal wall.

Birds lack an intromittent organ which is present in mammals. There is a small erectile phallus in the ventral part of the cloaca, which becomes erect when it is engorged by lymphlike fluid. Since the bird lacks accessory glands, the seminal plasma is produced primarily by the seminiferous tubules and the epididymis. There is also a transparent fluid, an exudate from the lymph folds in the cloaca, which is expelled when semen is collected from the cockerel. It is possible that some of this fluid is also released during the normal mating process. For a more detailed discussion of the reproductive tract in cockerels and the physical and chemical composition of seminal plasma the reader is directed to a fine review by Lake (41).

#### B. TESTICULAR STEROIDOGENESIS

The function of the testis is the production of spermatozoa and the secretion of hormones. These sex steroids are necessary for the growth

and maintenance of the accessory reproductive organs and regulate the courtship behavior. Estrogens, androgens, and progestins have been identified in the cockerel. Höhn and Cheng (29), reported high concentrations of estrone, estradiol, and estriol in the testis of fowls. These data are questionable because other investigators have found no evidence of estrogens in fowl testis (10). As in mammals, the identification and elucidation of the role of estrogen in the testis is a major question demanding an answer. Similarly, progestins have also been localized in the testis but it is not clear whether the progestins act locally, have some extratesticular site of action, or are present as precursors of testosterone (8, 10). Cardinali *et al.* (8) have identified testosterone as the important androgen in male fowl. The exact site of synthesis for testosterone is not clear because the enzyme  $\Delta^5$ -3- $\beta$ -hydroxysteroid dehydrogenase has been localized in both the interstitial tissue and germinal tissue of the testis (77).

In summary, it is necessary to identify what steroids are synthesized and secreted by the testis and to determine whether the interstitial and/or the germinal epithelium are sites of steroidogenesis. Hopefully, the cautious use of highly specific antisera against steroids may answer some of these major questions.

### C. CONTROL OF TESTICULAR FUNCTION

The testis in the chicken is not an autonomous organ but, as in mammals, the testicular function is regulated by the hypothalamus and pituitary. In 1959, Nalbandov (48) demonstrated that hypophysectomy of male fowl results in gonadal atrophy, regression of sex organs, and distinct changes in plumages. Later studies by Graber *et al.* (28) indicate that the gonadotropins appear to have distinct actions on the testis. While FSH is necessary to initiate growth of the seminiferous tubules, LH causes development of the Leydig cells and subsequent synthesis and secretion of the androgens. The action of prolactin in the male fowl is questionable. In an early study, Nalbandov (47) found that an injection of prolactin resulted in atrophy of the testis, decreased androgen production, and a reduction in comb size. However, some recent studies do not support these results as being applicable to all avian species.

In summary, it is readily apparent that the structure of the male reproductive system of the bird is strikingly different from that of the mammal. Despite this difference the interrelationships between the androgens and the gonadotropins appear to be similar in mammals and birds. Further investigation is required to define exactly what steroids are synthesized by the testis and where they are synthesized in the testis, and the function of these steroids in the cockerel.

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# 21 Nutrition and Reproductive Efficiency

P. V. Rattray

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## I. Onset of Puberty and Sexual Development

The age at onset of puberty can markedly affect an animal's reproductive efficiency, especially in the female. This is more important in the case of seasonal breeders where delayed onset of puberty and behavioral estrus could result in a year's delay in the production of the first newborn and so may reduce lifetime performance (4, 33). From a productivity point of view, the attainment of puberty in the first breeding season is less important in the male than in the female, but it could be important in selection programs by shortening the generation interval.

## A. SHEEP AND CATTLE

In sheep and cattle, size appears to be more important than age in determining the onset of puberty (3, 7, 37, 38, 81, 82, 88, 99, 109, 111). Different authors have postulated a threshold or genetically determined weight to be the main factor in the female (3, 81, 82). This is not as clear-cut in sheep as it is for cattle because of the seasonal nature in which they breed. The level of nutrition, by influencing growth rate and time taken to reach this weight, therefore affects the age at which these ruminants reach puberty. It is impossible to distinguish between the effects of level of nutrition on somatic growth and maturation independently from sexual development and maturation. Puberty occurs while the animal is growing and the sexual organs are functional before body growth is completed. In sheep it occurs at 40 to 70% of adult body weight, depending on the breed. Single ram and ewe lambs attain puberty earlier than twins and are heavier (37, 38). The restriction of energy, protein, or phosphorus delays the onset of puberty in heifers. Some animals, which lose weight over winter, fail to cycle by the end of the first winter (7, 81, 111, 142). Under very low levels of feeding, such as occurs in the tropics, some heifers do not exhibit heat until their third year of age (82, 160). In the male, deficiencies of protein, vitamin A, and zinc also severely impair the progress of sexual maturation (38, 109, 111).

If it is desirable to mate sheep and cattle early in life, relatively high feeding levels are recommended because there is a close relationship between general body growth and sexual development.

## B. PIGS

In contrast to the above species, the attainment of puberty in the gilt and young boar appears to be influenced more by age than by weight and occurs in most breeds at approximately 200 days of age (18, 141, 152). However, there are conflicting reports on the effect of level of either energy or feed intake on the age and live weight at puberty in the gilt (8, 18, 141). With mild restrictions of 60 to 70% of *ad libitum* intakes and even severe restrictions (50%) there have been reports of no effect, hastened onset, or delayed onset of puberty (91, 141). There are similar conflicting reports on the effect of protein levels in the diet. It appears that managerial factors such as the introduction of the male to the prepubertal gilt may exert a greater influence on age at puberty than does nutrition (18, 141). However, it is recommended to feed gilts so that normal growth rates are obtained. Although gilts are not usually mated at their first estrus, early

puberty, whether achieved by managerial or nutritional means, is important because ovulation rate is related to sexual age (number of estrous periods expressed prior to breeding), and early mating is desirable to increase overall productivity of the sow (18, 91).

### C. INFLUENCE OF NUTRITION ON ENDOCRINE FUNCTION AND SEXUAL DEVELOPMENT

In ruminants sexual development is highly dependent on rate of growth of the animal and reduced energy intake, as well as impairing body growth, impairs growth of the endocrine glands and reproductive organs (37, 38, 81, 111). There is good evidence that inadequate nutrition can have adverse effects on pituitary function, i.e., the synthesis or release of gonadotropins, and may also influence the response of target organs to gonadotropins or gonadal hormones (37, 38, 80). In malnourished ram lambs and bull calves, onset of the androgenic function of the testes is retarded more than spermatogenesis and this has been shown to be due to a lack of gonadotropins from the hypophysis rather than the inability of the testes themselves to produce testosterone (3, 38). In the severely underfed male the testes continue to enlarge and the seminal tubules appear normal but regression of the interstitial cells progresses until they become unrecognizable (81). Testicular histology in the ram is related to body weight (109).

In ewe lambs it has been shown that there is a close relationship between ovarian weight and body weight (37), and that lambs fed at a high level prior to puberty have larger reproductive tracts in relation to body size and have more multiple ovulations than poorly fed animals (81). In contrast, gilts that were fed at three different levels (100, 75, and 50% ARC recommended requirements) from weaning at 8 weeks to day 30 of their first pregnancy had larger anterior pituitaries, adrenals, and thyroids when fed at the highest feeding level. However, the relative weight of these organs as a proportion of body weight was lowest at the highest feeding level. The proportional weights of the ovaries and empty uteri were the same (66, 117).

Following severe undernutrition, optimal feeding of both males and females rapidly restores pituitary and gonadal function, and the development of the reproductive tract is accelerated. There is little evidence to suggest that full potential reproductive capacity is not completely recovered except for the occasional report with males, where optimal feeding did not result in complete recovery in size and function of the testes (3, 81). In this latter case, the underfeeding was much more severe than would normally occur in practice.



## II. The Influence of Nutrition on Reproduction in the Mature Female

Although specific deficiencies, imbalances, or the ingestion of toxic substances can influence various aspects of reproductive efficiency, either short- or long-term energy intake is of great importance. A reduced energy intake leading to a negative energy balance can lead to reproductive failure by influencing estrus, ovulation rate, fertilization rate, embryonic survival, prenatal losses, and birth weight (3, 82).

### A. ESTRUS, OVULATION, AND THE INTERVAL BETWEEN CONCEPTIONS

In sheep and cattle submaintenance feeding and emaciation may result in a cessation of estrus with a suppression of ovulation or ovulation without estrus (3, 50, 81). Mature animals are more resistant to dietary restriction than immature animals especially if the onset is gradual, in which case a major reduction in body weight may occur before anestrus ensues. It takes longer for an animal in good condition to become anestrus than one in poorer condition (81).

In sheep a high level of feeding, before and after parturition, reduces the postpartum interval to ovulation, and leads to more ewes cycling and being mated early in the breeding season (68, 89). The ovaries of well-fed lactating ewes contain mature follicles while the ovaries of poorly fed ewes remain inactive. Ewe live weight is significantly related to the postpartum interval to first ovulation; in heavy ewes the interval averaged approximately 20 days while in light ewes the interval averaged 40 days. This is especially important in accelerated lambing programs where a 6- to 8-month lambing interval is desired. A complex interaction between a low level of nutrition, lactation, and body condition and conception exists. The infertile interval following parturition may be due partly to a lack of sexual receptivity or short heats of low intensity rather than the absence of ovulation (68). In sheep, fasting has suppressed behavioral estrus (95), and feeding at maintenance or submaintenance has resulted in longer intervals between estrous periods and eventually to ovulation without estrus (81, 84).

Usually adult cattle are lactating when mated and the level of nutrition both before and after calving can influence the proportion of animals showing estrus and the interval between calving and conception (35, 56, 83). Live weight and live weight changes have been significantly related to ovarian activity and the occurrence of the first postpartum estrus (82, 160). Long-term weight loss in cattle has led to an increasing incidence of

ovulation without estrus and finally the majority of animals become anestrus (120). Low levels of protein intake have also led to an increased interval to first postpartum estrus (158). The dairy cow is a special case, in that during the first 2 months of lactation high producing animals are drawing on tissue reserves for milk production and, subsequently, are in negative energy balance (82). Lactation and low energy levels significantly delay postpartum follicular growth and ovulation (114). High producing cows even on liberal concentrate rations have a high incidence of cystic follicles and prolonged calving intervals (82). Occasional reports have indicated that high levels of feeding, compared with average levels of feeding, have led to ovulation without estrus, resulting in a longer interval to the first postpartum estrus (56, 164).

In pigs a postpartum estrus occurs in a proportion of sows within 1 to 3 days of parturition, but matings are usually infertile because ovulation does not occur. The sow returns to normal estrus 5-7 days after weaning (8, 18). High levels of intake and the feeding of sugar after weaning have advanced estrus (36, 103). Withdrawal of food and water for 24 hours immediately after weaning has in some cases, stimulated the onset of estrus (141). Level of intake and quality or quantity of protein during the previous gestation or lactation can influence the proportion of sows showing estrus and can influence the estrous interval (141, 149). The feeding of a protein-free diet has led to a cessation of cyclical activity in some animals (18, 118). Information on the effects of specific vitamins and minerals on this aspect of reproductive efficiency in pigs is limited but in gilts, diets low in manganese have led to depressed behavioral patterns at estrus or estrous failure. Vitamin A deficiency has resulted in irregular cycles but normal or intensified behavioral patterns at heat and normal conception rates occur (111).

In the mare,  $\text{CuSO}_4$  in the diet shortened the service postpartum period from 33.4 to 26.1 days, decreased follicle maturation time, and increased the gonadotropic potency of the serum (52, 112). Iodine supplements and injections of vitamins A, E, and possibly C have increased the proportion of cows coming into heat and reduced the interval to first-estrus (79, 161, 162). Phosphorus supplements and ingestion of phytoestrogens in legumes are reported to cause irregular cycling or reduce the number of animals showing estrus in sheep (42, 45).

## B. OVULATION RATE

Both long-term levels of nutrition (and its effect on body weight) and sudden changes in the level of nutrition at or before mating can influence ovulation rate. These two aspects can have complex interactions and

there are many variable reports on the effects of nutrition on ovulation rates. In a recent review, Lamond (82) stated that in litter-bearing species, such as pigs and mice, sudden increases in the level of nutrient intake led to increases in ovulation rate. In species such as sheep and goats, where a variable proportion have two or three ovulations, the ovarian response to sudden increases in nutrition is not as consistent and live weight per se may be more important than live weight gain, but both are involved in a complex relationship. In contrast, for the uniparous species such as the bovine, live weight may be the only important variable.

## 1. Pigs

There are several recent extensive reviews on the influence of nutrition on ovulation rate in pigs (8, 18, 141). In the latter report nutritional effects are discussed under three sections depending on the length of feeding period: long-term, within 21 days of ovulation, and single-feed flushing near ovulation. "Flushing" is the term used for increasing the level of nutrition prior to mating (81). If gilts were fed diets *ad libitum* for a long period before puberty, ovulation rates were generally higher than for gilts fed at restricted levels. However, age of the gilts was a complicating factor. In sows responses have been more variable, and there may be a carry-over effect from the previous gestation or lactation on body condition of the sow. There have been reports of 0.9 fewer corpora lutea for every 10 kg live weight loss in the sow during the previous lactation (8, 141). Increasing the level of nutrition for periods of up to 21 days or even 4-6 days immediately before estrus generally results in an increased ovulation rate (8, 18, 19, 23, 29, 141). There have been some reports of success in increasing ovulation rate or litter size by increasing the level of feeding of gilts on the day of estrus but not on the day following estrus (19, 141). Other similar experiments with gilts and sows have met with little success. Body condition may be important in whether a flushing response is obtained; thin, but not fat sows, have given a positive response in ovulation rate to increased feed intake (141).

It is the energy component of the ration that is important and responses have been obtained from supplements such as glucose, sucrose, corn oil, or lard. It has been suggested that an increase of 6 to 8 Mcal of metabolizable energy per day is sufficient to stimulate the increased ovulation rate. The optimum duration of the high energy regime is 11-14 days before mating (8). It appears that the level of energy intake during the follicular phase of the cycle is the important factor, as increases in intake in the first half of the cycle have not increased ovulation rate (18). Starvation for 10 days prior to estrus and for periods as short as 48 hours following signs of estrus

have led to lower ovulation rates when compared with *ad libitum* feeding. The hydrazine derivative, methallibure (ICI 33828) may block the mechanism whereby high energy levels increase ovulation rate in pigs (144). This compound is a hypothalamic inhibitory drug and inhibits the release of LH and FSH in swine. Quantitative changes in the LH and FSH content of the pituitary and progesterone content of corpora lutea dependent upon energy intake have been reported in gilts (144). Whether the increased energy level acts directly on the pituitary or via some feedback mechanism has not been shown. Full feeding during the last 4–6 days of the cycle have led to an increase in follicle weight and the number of large follicles, and a decrease in the ratios of small:medium follicles and medium:large follicles (29). High levels of feeding have led to increased ovulation rates and heavier anterior pituitary glands (23).

Protein intake within reasonable limits does not usually affect ovulation rate (18, 31). Occasionally higher protein content of the diet or amino acid supplements may enhance intake and ovulation rate in gilts (18, 149). Although deprivation of protein for one cycle has had no significant effect, prolonged deprivation (four to six cycles) has led to a significant reduction in ovulation rate (18).

## 2. Sheep

Ovulation rate and lambing performance in sheep have been improved both by flushing and live weight *per se* at mating (15, 22, 24, 49–51, 57, 67, 82–84, 152) and these have been termed the “dynamic” and “static” effect, respectively. In some reports of improved lambing performance it is difficult to ascertain whether this was due to an increase in ovulation rate or some other aspect of reproductive efficiency. An increased twinning rate of 5 to 20% for every 5 kg increase in live weight at mating has been reported (5, 22, 24, 34, 49, 65). Differences have been explained on the basis of age, breed, and season (5, 22, 24). The relative contributions of skeletal size and body condition to this live weight effect have not been defined (15), but body condition score has been positively related with ovulation rate (62). Flushing increases ovulation rate by as much as 10 to 20% (15, 24, 152, 155), but body condition may affect the response to flushing, as there have been reports of lack of response from ewes in good condition (152) or a positive response only from thin ewes (61). There is much disagreement in the literature over the relative importance of body weight (or condition) and the flushing effect. Interactions are complex, and it is difficult in many cases to separate one effect from the other. It has been found that flushed ewes have higher ovulation rates than those maintained in high condition (152). In contrast, other workers found no

significant difference in ovulation rates between two groups of ewes of similar body weights that were either gaining or losing weight (26). Ewes transferred to submaintenance diets have continued to have high ovulation rates until considerable weight loss had occurred (152). Reports of the flushing effect have been less consistent than reports of the live weight effect, so the latter may be more important in determining ovulation rate in sheep. It has been stated that flushing per se did not influence ovulation rate, but that the increase in live weight brought about by flushing was the responsible factor (49). Whatever the precise reason, increasing the level of intake or feeding concentrates prior to mating, has increased ovulation rate and the level of response appears to be influenced by the duration of the increased feeding (24, 57, 83, 152). Periods of 3 to 4 weeks or longer are recommended. Shearing prior to mating may promote intake and cause a flushing effect (68).

Unlike findings with swine, no particular part of the estrous cycle has been shown to be critical in respect to flushing and short periods of flushing prior to estrus have not had any effect (24, 83). Recent work suggests that the flushing effects on ovulation rate may be greatest early or late in the breeding season, but that at the peak of the season, feeding level may have very little effect (67).

Flushing responses have been obtained to both energy and crude protein levels in the diet. Concentrate supplements of grain, starch, and fat have all proved efficacious and thus the energy level is probably more important (6, 57, 102, 152, 155). The higher ovulation rates have been associated with larger follicles and corpora lutea, increased follicular fluid weight, and a higher ratio of large:small follicles (6, 152). Heavier pituitaries have also been reported (and hence gonadotropin potential) but there was no increase in gonadotropin concentration in the pituitary gland (152). The actual nutrients responsible for the flushing have not been isolated and the actual mechanism, whether acting directly on the pituitary or via feedback mechanisms, has yet to be elucidated.

The age of the ewe may influence her response to flushing as yearlings appear to respond to a lesser extent than mature ewes, and flushing appears to have no clear effect in ewe lambs (37). Mating of ewe lambs (at 7 to 9 months), however, may reduce live weight and subsequent fertility at 18 months, but not in later years (75, 159). Long-term carry-over effects have been shown where severe undernutrition up to 15 months of age has led to a decrease in the proportion of twins at the third, fourth, and fifth lambings (127). Very severe undernutrition of mature ewes (5½ years old) during the spring and summer have led to depressed ovulation rate the following autumn, even after body weight loss had been regained, but it was not known whether the effect was permanent or temporary (50).

## Cattle

The bovine is predominantly a single ovulating species and nutritional manipulation of ovulation rate cannot be considered (15, 82). However, as will be discussed below, nutrition and live weight can affect aspects of fertility in the cow.

## CONCEPTION AND EMBRYONIC MORTALITY

There is considerable evidence that specific nutrients, level of feeding, and/or body condition may influence the fertilizability of ova or ease of conception and embryonic survival in most species of domestic and laboratory mammals. To quote Edey (39) in a recent review of this topic in sheep: "Little is known of the reasons why apparently normal fertilized embryos develop for a period and then die." The term "hostile uterine environment" is often used but the actual cause is unknown: whether due to uterine secretions or enzymes or to endocrine interaction. In the multi-ovulating species, ovulation rate alone (and hence plane of nutrition before mating) may influence embryonic mortality (39, 141). There is some difficulty in ascertaining whether reproductive failure around the time of mating is due to lack of fertilization or to embryonic loss.

## Pigs

In the gilt a high level of feeding or of energy prior to mating is often associated with an increase in embryo mortality (14, 18, 53, 54, 140). Most losses appear to occur between days 9 and 25 of gestation, but some fetal mortality occurs after day 60 when fetal growth starts to accelerate (141). In gilts, embryo loss increased with increasing feeding levels after mating and this loss was independent of the premating feed level. Conversely, a reduction of energy intake or short-term fasting after mating improved embryo survival. In contrast to gilts, level of feeding has little or no effect on embryo mortality in the sow (18). Minimal levels of feeding which affect litter size have not been established clearly. In most, but not all, reports there have been no deleterious effects from intakes as low as 3.0 Meal digestible energy per day (18, 53).

The mechanism by which energy level influences embryonic survival has not been discovered, but in reciprocal ova transfer experiments embryos were found to be equally viable, suggesting that some maternal or uterine factor was involved (14).

In laboratory animals protein restriction can increase embryonic losses

at the time of implantation, by affecting placental development and up-setting nidation (104, 136). In pigs, protein quantity and quality are relatively unimportant in respect to reproductive performance of gilts or sows (18, 73, 118). However, there are occasional trials where conception rate, fetal resorption, or litter size may have been affected (63, 115).

Because most diets for swine are formulated as balanced rations, reports of mineral or vitamin deficiencies are rare, but there have been positive responses reported in litter size to Ca, Zn, Se, choline, and vitamin A (96, 121, 147, 163). Increases in conception rate and/or litter size have been obtained from feeding antibiotics (39, 131); negative responses have been obtained with rapeseed meal, probably due to the presence of a goitrogen (32).

## 2. Sheep

Experimental results on this subject are very variable and often contradictory. There is some evidence that level of nutrition or live weight affects fertilization or ease of conception. The majority of work suggests that higher levels of feeding (both before and after mating) or heavier ewes result in improved conception rates (24, 30, 51, 57, 152), but some studies have shown that flushed ewes are slower to conceive (152) and that ova from high plane ewes have a lower fertilization rate than those from low plane ewes (82). Previous level of nutrition appears to be important in that good levels of feeding for 4 weeks before or 3 weeks after insemination improved fertility only in ewes that were previously moderately fed, but had no effect or a negative effect in those that were well fed previously (56).

The effects of nutrition on embryonic survival are equally confusing and, again, experimental results have been extremely variable. It appears that high levels of feeding, concentrates, high live weight, and good body condition (irrespective of feeding) may all lead to increased embryonic loss (39, 57, 62, 152). Again, this appears to be influenced by previous feeding levels, in that lower losses may be obtained if previous feeding levels were moderate, but no response or higher losses may be obtained if previous levels were high (56). There have been many reports of no response, and they have varied from year to year and may be different between breeds. On the other hand, several reports suggest that in some circumstances low live weight, submaintenance feeding, or short periods of starvation may also increase embryonic losses (26, 27, 39, 152, 163). In one study, ova from poorly fed ewes were readily fertilized but the zygotes were of low viability or the uterine environment was unsatis-

factory for survival (82). The embryo loss appears to be sensitive to level of nutrition up to about day 26 of gestation only (39, 62) and, in some studies, as the period of restriction increased so did the incidence of embryonic loss (27). Younger ewes, fat ewes, and twin ovulating ewes appear to be most sensitive (26, 27, 39).

The situation is very complex and is confounded because high levels of supplementation and high live weight, while they augment ovulation rate, may also increase embryonic loss, and these two factors themselves may be related. Because of the apparent risk also of nutritional restriction after mating, a moderate or maintenance level of feeding is recommended at this stage. Synchronization of estrus would be a useful aid in implementing the efficiency of feeding the breeding ewe (152).

Some specific factors are also associated with conception and embryonic loss in sheep. Supplements of trace elements Cu, Mn, Zn, and Se have improved the percentage of ewes that lamb (40, 137). Estrogenic legumes have been associated with reduced conception rate, increased embryonic losses in the first 20 days, and also fetal losses from day 60 to term (30, 39, 45). In estrogenic cultivars (varieties) of subterranean clover, conception rate was significantly related to the content of the isoflavone, formononetin (30). This infertility problem may be partially reduced by treatment with Se (58). Goitrogens in forage crops such as kale or rape and supplements of phosphorus have also been associated with increased embryonic losses (39, 42). The feeding of lupines before mating has also improved the number of ewes lambing and this was probably a response to protein (77).

### 3. Cattle

Fecundity in the bovine cannot be influenced by nutrition but fertility can be affected by both long- and short-term levels of nutrition, as well as the supply of specific nutrients. Both fertilization and embryo survival appear to be influenced.

Nutrition over an extended period and its effect on body weight or body condition appears to be the major criterion influencing fertility in the cow (9, 82, 152, 160). Weight loss over winter before the previous calving can have carry-over effects resulting in lowered conception rates. Up to 10 or 15% weight loss can be tolerated at this time without adverse effects provided the cows are well-fed from calving to mating (82, 138, 152, 160). On the other hand, obesity is detrimental to fertility (10, 82, 111, 119).

Nutrition over short intervals is also important, in that cows gaining weight prior to mating have higher conception rates than those maintaining



or losing weight (82, 100, 107, 138, 152, 160). Lactational stresses, especially in the dairy cow, can lead to increased inseminations per conception (82, 99, 152). Short-term increases in the feeding of cows prior to mating may be of limited value if cows are in poor condition, and it may be wasteful if cows have reached a body weight or condition capable of conception (82). Mainly, this conception rate response is due to level of feeding, feed quality, or the supply of carbohydrate. Low conception rates have been associated with hypoglycemia and this has been ascribed to an energy deficiency (82, 99, 100). In the tropics or arid regions, feed quality may be the major factor influencing conception (82, 160, 165). Underfeeding of the cow between calving and insemination can result in both infertility and increased embryonic losses (56). Short-term submaintenance feeding of heifers has resulted in decreased plasma progesterone levels, reduced numbers and size of follicles, poor fertilization of ova, and reduced weights of corpora lutea (82). Plane of nutrition can influence the ovarian response to gonadotropins and a 72-hour fast after PMSG injection has led to reduced follicular development (82). Similar to findings with sheep, there are some reports where concentrate supplements after insemination have reduced losses if preexisting levels of feeding were low, no effect if the level was balanced, and increased losses if the preexisting level was excessive (56). This latter observation may account for some of the reports where more services per conception were required for heifers raised on high planes of nutrition (152).

There have also been reports of low conception rates where crude protein levels have been very low and these have been improved by supplementation (99, 135, 158, 165). Responses have also been obtained from high quality protein supplements, but these may have been associated with improvements in body weight and condition (13). Feeding of excess protein has also lowered conception rates, probably from increased embryonic losses after insemination (55, 56, 128).

Water restriction after mating, with its subsequent dehydration and weight loss has decreased conception rates substantially (71).

In deficiency circumstances, supplements of various minerals, trace elements, or vitamins have improved conception rate or fertility in both cows and mares. There have been responses to P, Ca, I, Cu, Zn, Co, and Mn, plus vitamins A (or carotene), E, and C (20, 79, 99, 101, 128, 135, 145, 152, 160-162, 165). In some reports combinations were administered and it was then difficult to tell which nutrient induced the response.

The following feed factors or nutrients in excess have been associated with depressed reproductive performance in cattle; goitrogens (in cruciferous crops and legumes), phytoestrogens (in legumes), nitrates, thyroprotein, Na, P, Ca, and F (99, 128, 139, 152).

### III. Influence of Nutrition on Reproduction in the Male

While information for the stallion is almost entirely lacking, the mature male of the other domestic species is remarkably resistant to nutritional stress and, in practice, male infertility problems of nutritional origin are seldom encountered (81, 111). Some adult bulls and rams have produced sperm right up until death (152). Younger males are more susceptible to nutritional stress than adult males (81, 152). Severe undernutrition and vitamin A deficiencies are the two most common causes of impaired reproductive capacity in the male.

#### A. LIBIDO

Libido is an important aspect of male reproductive efficiency as it influences the coverage of estrous females (97) and this could be critical with sheep and cattle in the field, when large numbers of females are cycling at the peak of the breeding system. It could also be extremely important where modern research is advocating much lower ram/ewe ratios (4).

Libido is affected before spermatogenesis, probably due to depressed androgenesis (3, 38, 97, 152). In rams, submaintenance rations have led to depressed libido within 5 to 10 weeks and the decline progressed as underfeeding continued (16, 97, 116). Undernutrition similarly affects the bull (87, 111, 152). In some cases the decline in libido may have been due to physical weakness (152). Long-term vitamin A deficiency has led to decreased sexual activity in both rams and bulls (152). Because of liver reserves of vitamin A it often takes 5–6 months of feeding a carotene-deficient diet before the symptoms are manifest (109). Overfeeding and obesity can also lead to a loss of libido especially in hot weather (99, 165). In Europe on trace-element deficient soils, supplements of I, Co, Cu, and Zn have led to increased sexual activity in bulls (2). There are very few reports of depressed libido in boars (111, 152).

#### B. SPERMATOGENESIS AND SEMEN QUALITY

Prolonged undernutrition has led to a depression or cessation of spermatogenesis in boars, rams, and bulls (17, 99, 111). This is often accompanied by a decrease in the size of the testes and atrophy of the interstitial cells (109, 111, 158). With rams on a restricted energy intake, the decrease in testicular weight was accompanied by decreased testicular blood flow, oxygen uptake, and glucose uptake (109). After prolonged undernutrition in all three species, semen quality was adversely affected

most commonly with decreases in: ejaculate volume, sperm production (density and total numbers), live sperm, motility, freezing resistance, viability and survival, and increases in abnormalities (86, 87, 99, 109, 111, 152, 158). Optimal nutrition appears to reverse the changes successfully (111). Not all the reports of poorer semen quality are accompanied with reports of poorer fertility, but in many cases changes in conception rate or fertility have been recorded (87, 152). In spite of these variable reports this could be quite important as fecundity of ewes has been influenced by semen quality of rams (90); and in swine using artificial insemination the fertilization rate of ova increased as the numbers of sperm increased (1).

Apart from the effect of undernutrition on sperm production, the main effects may be on the accessory genitalia affecting the composition and biological properties of seminal plasma; the production of fructose, citric acid, and other secretions can be markedly affected (81, 86, 109, 152). This is a consequence of the reduced testosterone secretion that occurs (38, 109, 135).

Most of the above responses are due to level of feeding or energy (109, 116, 158). It appears that if sires are not in good body condition, supplementary feeding 2 months prior to breeding may improve their performance over the subsequent mating period by virtue of a beneficial effect on libido and sperm production, as it takes approximately 50 days for the gamete to develop, mature, and appear in the ejaculate (116, 165).

Quantity or quality of protein appears to be relatively unimportant in its effect on spermatogenesis unless it results in depressed food intakes (72, 111, 152). Some responses have been reported in sheep and cattle especially where energy intake is inadequate (97, 111). Boars have responded in increased sperm production and quality (and subsequent fertility) from supplements of lysine and methionine (113, 146, 153).

Vitamin A deficiency inhibits spermatogenesis and adversely affects semen quality and fertility in a fashion similar to undernutrition (12, 99, 109, 111, 152). Degeneration of the germinal epithelium, reduced diameter of the seminiferous tubules, and testicular atrophy are common symptoms and these are usually accompanied with cystic changes in the pituitary (111, 152). These signs of damage can exist for long periods after supplementation with carotene. The pituitary changes suggest malfunction of gonadotropin production or release, but intratesticular application of vitamin A has restored spermatogenesis in surrounding tissue. These pituitary changes have not been found in swine, but this may only indicate degree of deficiency (111). Supplements of vitamin A and C have led to improved sperm production, semen quality, and conception rates in swine, the latter especially in hot weather (70, 166). Vitamin E and fatty acids are required for testicular integrity in small animals but no spe-

cific deficiency symptoms related to these nutrients have been found in farm animals (98, 111). The feeding of fat-free diets to boars, however, has led to a decline in testicular weight and spermatogenic activity (152). This could be due to a deficiency of either the fat-soluble vitamins or essential fatty acids.

There have been responses in sperm production, semen quality, and fertility in sheep and cattle to the following trace elements: Cu, Co, Zn, Mn, and I; and responses in semen quality to I in stallions (11, 20, 109, 116, 133, 148, 158).

Polyphenol oxidase supplements in the feed have improved semen quality and fertility in boars and bulls (143, 158). Feeding of excess thyroprotein or goitrogens to rams has lowered semen quality (152).

#### IV. The Influence of Nutrition during Pregnancy on Development and Survival of the Fetus and Neonate

The most recent and comprehensive reviews on the nutrient requirements for pregnancy in domestic animals are those by Lodge (92, 93). Moustgaard in the first two editions of this book reviewed the patterns of nutrient deposition in the products of conception for the porcine and bovine (110, 111). These were based on chemical analyses of fetuses, fetal membranes, fetal fluids, and uteri. Similar data now exist for the ovine where the pattern of retention of some major minerals and other components have been obtained by analyzing the products of conception at various stages of gestation (48, 85, 125, 129). Data for trace elements are not available at this stage. Except for dietary energy and protein there is very limited information on the utilization of other nutrients for pregnancy. Sheep and cattle utilize metabolizable energy for fetal growth with a net efficiency of 10 to 14% and for conceptus plus mammary development of 16 to 24% (94, 106, 123, 124, 150). Because the fetus requires energy in the form of glucose, energy utilization for fetal growth is probably less efficient in ruminants than in pigs because the final products of ruminant carbohydrate digestion are volatile fatty acids rather than hexoses (93). In sheep, especially those on restricted feeding, body protein is used for glucogenesis and this contributes to the heat increment of pregnancy (60, 93). Swine may also mobilize tissue protein for conceptus development (76). In sheep, protein is utilized with an efficiency of 20 to 30% for conceptus growth (150). Protein and amino acid requirements for pregnancy in swine were given recently (41). It is outside the scope of this chapter to list detailed requirements for pregnancy, but they are readily available from the above reports. In the bovine the situation is compli-

cated by the fact that usually the requirements for pregnancy and lactation overlap (93).

### A. PRENATAL GROWTH AND DEVELOPMENT

Fetal growth follows an exponential pattern, but the basic instantaneous growth rate decreases as parturition approaches (78, 125). The fetus, fetal membranes, and fetal fluids change as relative proportions of the conceptus (Fig. 1) as gestation progresses (125, 134). Placental development is virtually completed by the end of the second trimester of pregnancy, while 80% of fetal growth occurs during the final trimester (44, 111, 125, 129, 134). Nutrient requirements for the fetus follow a similar trend to fetal growth, being very low in early pregnancy and increasing markedly in the last trimester. For example, total energy requirements rise during late pregnancy until they are 50–100% greater than the nonpregnant maintenance requirement (93, 105, 124, 130).

Undernutrition, in terms of energy or protein level, in late pregnancy

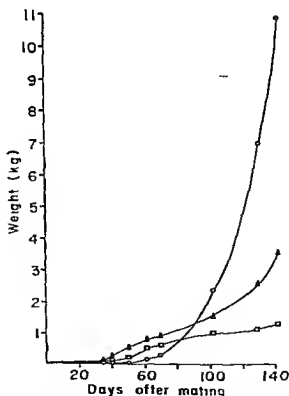


FIG. 1. Weights of both fetuses, membranes plus placenta, and fetal fluids at different stages of gestation for the ditelous ovine. Fetuses, —○—; membranes plus placenta, —□—; fetal fluids, —△—. From Rattray *et al.* (125) and Rattray (unpublished).

can result in a substantial depression of birth weight, and this is associated with increased perinatal losses (21, 44, 54, 93, 111, 115, 134, 152, 157) Shearing of ewes in late pregnancy has led to higher birth weights, possibly by stimulating intake (132) Underfeeding during early and mid-pregnancy generally has had little adverse effect on fetal development and birth weight, if the ewe is well-fed in late pregnancy (25, 108, 111) Nutritionally restricted fetuses have demonstrated compensatory growth and development when given the opportunity to do so (44) Maternal undernutrition in early and midpregnancy has led to restricted placental development in sheep, and this could limit potential fetal growth in late pregnancy (43, 44) Underfeeding of ewes and cows has shortened gestation (44, 152, 157) Ad libitum fed ewes also have had shorter gestations but their intakes declined in late pregnancy (152) This decline in intake of well-fed ewes in late pregnancy has been commonly encountered in twin bearing or fat ewes, and this may be due in part to the increase in uterine volume leading to physical reduction of rumen capacity and to some metabolic factor (43, 46, 47, 125) Birth weight and gestation length have been found to be highly correlated with live weight at mating in multiparous ewes (74), but not in single bearing ewes (43)

Energy deficiency in late pregnancy can result in pregnancy toxemia in sheep with the resultant loss of both ewe and lamb (93, 111) Excessively high levels of feeding can lead to large single fetuses with an increase in lambing difficulties and lamb mortality associated with dystocia (28, 64) In swine, excessive feeding during pregnancy can be wasteful and may impair parturition (21), but feeding of pelleted diets has increased birth-weight (154)

Adequate supplies of minerals and vitamins during pregnancy are necessary for normal development of the fetuses (111) Several minerals have been shown to affect fetal development The stage of gestation at which a deficiency occurs influences its manifestation (69, 111) Depending on the severity of the deficiency, if it occurs in the first third of pregnancy when organogenesis is sensitive, structural abnormalities or fetal death can occur, if it occurs in the last two-thirds of prenatal life, functional abnormalities, death, or nonviable offspring may result (111) Of the major elements, Ca has received the most attention and its deficiency leads to increased stillbirths (93, 111) Vitamin D supply may influence this Even though a shortage of P may impair estrus and ovulation, it appears to have little effect on pregnancy per se Iron deficiency will lead to anemia in the mother, but while fetal hemoglobin levels appear normal, it may predispose the neonate to anemia In swine, especially, the hemoglobin level of the suckling new born falls, and exogenous Fe in addition to that in the milk needs to be supplied Attempts to elevate fetal Fe levels by increased

maternal supplies have been unsuccessful (93, 111). Iodine deficiency or ingestion of goitrogens lead to fetal death, abortion, congenital goiter, lowered birth weights, and debility of the neonate (32, 85, 93, 111, 152). Manganese and Zn deficiencies are associated with several abnormalities, such as, gross fetal malformations, reduced birth weights, fetal resorption, and stillbirths (69, 93, 111, 152). Copper deficiency influences fetal development with the birth of weak, ataxia offspring (93, 111, 152). This may, in ruminants, be aggravated by excess dietary Mo (111). Selenium deficiency has also led to an increase in fetal resorption in pigs (96). Excess amounts of S, Na, and NO<sub>3</sub> have caused abortions in sheep (39, 93, 102, 119, 152). Phytoestrogens have also appeared to be associated with increased losses of ewes and lambs at parturition (152).

Deficiencies of vitamins E and K have caused fetal resorption in laboratory animals but there appears to be no relevant data for farm livestock (93, 122). Shortages of several of the B complex vitamins have been implicated in causing congenital abnormalities and fetal resorption in pigs and rats (93). There is a well-established relationship between vitamin A supply and fetal development in most species. A deficiency will cause congenital malformations, abortion, retained placentas, an increase in stillbirths, and debilitated young (93, 152). In pigs there has been a response in birth weight and numbers born with supplements of this vitamin (59), but in rats an excess intake has led to fetal malformation (111, 113). Choline levels have influenced the number of live pigs per litter (147). Vitamin D deficiency, because of its close association with Ca and P metabolism, can lead to the birth of rachitic young (93).

## B. THE EFFECT OF PRENATAL NUTRITION ON POSTNATAL SURVIVAL AND DEVELOPMENT

The effect of undernutrition in late pregnancy can influence early postnatal survival by, either directly affecting the neonate or by carry-over effects on the dam. The reduction in birth weight is commonly associated with impaired neonatal vitality (44, 93, 111, 152). A good example is in lambs, where there is a high incidence of mortality in very light animals (64, 152). Underfeeding of ewes in late pregnancy has led to light lambs with lower fat depots and less liver glycogen and, because of their limited energy reserves at birth, and their relatively high surface area to body mass, they succumb more easily to exposure in inclement weather; the resultant loss of suckling drive may cause physiological starvation. This is enhanced by delayed onset of lactation and restrained behavior of the dam (44, 156).

Frequently the young from mothers that were poorly fed during gesta-

tion have slower postnatal growth rates, and differences in birth weight may be magnified by weaning. Slower postnatal growth is usually associated in part with reduced milk production of the mother. Part of the cause of these lowered growth rates could have been due to reduced numbers of fibers in skeletal muscles (44). This could be of major concern in meat-producing animals. In a recent study with the ovine, on hyperplastic and hypertrophic growth of early, intermediate, and late developing tissues, it was shown that cellular hyperplasia of early developing tissues was still evident near term (126). Nutritional stresses can have irreversible effects if imposed during cellular hyperplasia, so adequate feeding in late prenatal and early postnatal life is essential. There have been reports of permanent effects on mature weight and wool production from severe undernutrition during pregnancy in sheep (44).

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# 22 Genetic Variation and Improvement

R. B. Land

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## I. Introduction

The creation of new individuals which resemble their parents is the essence of reproduction, and genetics, the study of resemblance, is the concern of this chapter. The genes which an individual inherits from its parents normally determine the general form of an individual, including its reproductive characteristics. An understanding of the inheritance of reproductive performance therefore offers the opportunity for genetic improvement, improvement which is permanent in the sense that it is not dependent upon potentially costly modification of the environment through the use of husbandry, nutritional, or pharmacological manipulations. Genetic improvement is, however flexible in the sense that, providing variation is conserved, it still offers the possibility of further change should objectives or circumstances alter. It is the potential to combine permanence with flexibility which is the virtue of genetic improvement. Just as the genes an individual inherits influence its reproductive performance, so the reproductive rate of a group of individuals may, in turn, influence the

possibility of genetic change for it affects the number of individuals available for selection.

The physiological study of genetic variation may indicate which physiological component is limiting the output of a particular system; and, as a result, it may be possible to define a physiological criterion for genetic selection to improve the output from that system. The quantitative physiological expression of genetic variation is, therefore, of particular concern.

This chapter does not claim to be comprehensive, for little space has been found for variation in male performance, perinatal mortality, or for reference to the extensive literature on birds, laboratory animals, and man. The emphasis is on quantitative variation and the improvement of domestic mammals. Within these limits, I hope to deal with some theoretical and practical aspects of genetic variation in reproductive performance.

## II. Determination of Sex

In mammals, male and female gametes are produced by different individuals; sexual reproduction is obligate. Fertile males and females would be favored by natural selection, and deviations from normality progressively eliminated. With little variation in sexuality within sexes, the hypotheses regarding the inheritance of sex in mammals are largely based on extrapolation from lower animals, and from the study of abnormalities (5, 15, 43, 46, 58). This account and the summary (Fig. 1) are based on these publications.

During embryogenesis, the gonads develop from the genital ridges colonized by primordial germ cells which have migrated from the region of the yolk sac. There are, therefore, two sources of the determination of sex: the germ cells and the gonadal stroma. A third component is recognizable as the ability of somatic cells to respond to gonadal activity in the determination of secondary sexual characters.

### A. GONADAL AND GERM CELL SEX

In the presence of a Y chromosome, the medulla of the embryonic gonad normally develops to form a testis, whereas in the presence of two X chromosomes the cortex normally develops to form an ovary. In the presence of two X and one Y chromosome the Y is dominant to the extent that a testis is formed and the phenotype is male but sterile, indicating the overriding influence of the Y chromosome on gonadal and phenotypical sex. This can be mimicked by autosomal genes (e.g., the intersex goat and sex-reversed mouse) which effect the development of a testis, secreting

androgens, in the presence of two X chromosomes and no Y. Only the gonad is sex reversed by these genes; the primordial germ cells enter meiosis as primary oocytes, but progress no further. The sex of the germ cells is independent of that of the gonads.

Anomalies may also operate in the other direction, e.g., testicular feminization, when an XY genotype is accompanied by small testes with no spermatogenesis but showing a relatively normal female phenotype. The gonad, however, is not "reversed," the action of testosterone on somatic tissues is blocked, and female characteristics develop in response to testicular estrogens. The blocked response to testosterone also reduces hypothalamic feedback (24).

The presence of a single X chromosome leads to the formation of an ovary in several species, but with the exception of the XO mouse, oogenesis is blocked, indicating that two X chromosomes are normally required for the development of ova. In addition to sterility, normal endocrine activity of the ovary is absent.

The genetic abnormalities recorded, therefore, concern the development of testes in unusual circumstances, but not the formation of an ovary in an "XY environment." This virtual unidirectional modification has led to the argument that the female gonad develops in the absence of modification in a male direction. The same regulatory mechanisms may control the development of both sexes, testosterone inducing maleness (47), but Mittwoch (43) argues that the basic difference is one of growth rate at the time of differentiation. More recently, the first sign of sexual differentiation has been reported to be the development of characteristic cell surface antigens (2, 66), which may lead to immunological methods of sex selection.

## B. STEROID SECRETION AND SECONDARY SEX

Somatic tissue is apparently sexually undifferentiated; both overt secondary sexual characteristics and the "sex of the hypothalamus" are influenced by fetal steroid production. Testicular feminization demonstrates the ability of XY somatic cells to develop into a female which is apparently normal externally. The sex-reversal genes show that XX somatic cells can show male characteristics. Furthermore, testosterone eliminates or reduces hypothalamic cyclicity so that hypothalamic sex is a function of gonadal sex (59).

The central role of the gonadal steroid hormones in the determination of both hypothalamic and external secondary sexual characteristics may have led to the clear differences in steroid production between normal males and females. In particular, there must have been intense selection

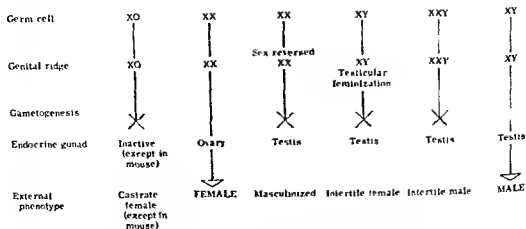


FIG. 1. Summary of the effects of genetic abnormalities on sexual differentiation.

for the reduction of testosterone production by the female, for this hormone would have destroyed the essence of femaleness, hypothalamic cyclicality, positive feedback and, in due course, ovulation—possibly through changes in the balance between the conversion of androstenedione to testosterone or estrogens.

The genetic determination of sexual and asexual phenotypes is summarized in Fig. 1. The sex of the gonad influences external phenotypical sex, but not the sex of the germ cells. Germ cells, however, only develop in a gonad of the same sex, and in the female two X chromosomes are usually essential for oogenesis. Secondary sexual characters are dependent upon gametogenesis in the female but not in the male. The role of the autosomes and the number of genes involved in sex determination remain to be resolved.

Most attempts to artificially influence sex ratios have considered the separation of X and Y carrying sperm with little consistent success. It may be fruitful to consider the modification of differentiation following fertilization, either physiologically or immunologically.

### III. Variation among Species

Females of different cutherian mammalian species give birth to litters which vary in weight by a factor of approximately 10%, from as much as 1 metric ton in the case of some whales to as little as 2 gm for the shrew, a clear example of genetic variation in reproductive performance within the female sex. This variation is associated with differences in body weight, and it has been found that  $\log \text{ litter weight (gm)} = 0.8 \log \text{ maternal weight (gm)} - 0.33$ , (40) so that, in general, litter weight would be expected to vary around an average value of 50% of maternal weight to the



power 0.8. One of the consequences of this relationship is that as maternal weight increases, litter weight as an absolute proportion of maternal weight will decrease. The weight of individual young, however, as a proportion of maternal weight is independent of maternal weight, varying around an average of 5% (Taylor, personal communication). Among species, one of the consequences of litter weight being related to a lower power of maternal weight than is individual birth weight is that the number in a litter must fall as maternal weight increases, and, indeed, such a relationship may be thought of as common knowledge.

A fascinating range of variation in reproductive performance has therefore evolved. However, it is not haphazard and conforms to a basic pattern. Some aspects of this pattern developed during many discussions with Dr. St. C. S. Taylor are summarized below.

Perinatal mortality tends to increase when individual birth weight exceeds 10% of maternal weight due to difficulties at parturition, and when it is below 3% of maternal weight due to neonatal morbidity. The present reproductive system of mammals, therefore, determines their maximum size such that when the total weight of the litter decreases below 3% of maternal weight, then, even with the production of only one young, it is at the limits of survival (i.e., when adult weight increases beyond the point when 50% of adult weight to the 0.8 power is less than 3% of adult body weight). The superimposition of a 5% mean on the ratio of litter weight to adult weight (Fig. 2) shows that the largest mammals lie where the two lines meet. With lower maternal body weights, once 5% of maternal weight is greater than 50% (maternal weight)<sup>0.8</sup> multiple births could be favored.

Given the evolution of the present pattern of mammalian reproduction, natural selection has led to a negative association between litter size and maternal weight. Why then is the reverse relationship present within species, where larger strains tend to have larger litters? The answer to this paradox may be in the duration of pregnancy. The gestation length of a species is positively related to adult body weight—the fetuses of larger species spending a longer time to grow from conception to around 5% of their adult weight than those of smaller species. A species, however, is a potentially interbreeding group, so that within a species the duration of pregnancy is relatively constant. With limits to fetal growth in a given period of time, large strains within a species would give birth to relatively small young and small strains to relatively large ones. This is borne out by the literature (12, 61). The range of body weight between strains within a species will, therefore, be restricted at the upper end of the distribution by the ability of the fetus to grow fast enough for it to be more than around 3% of maternal weight, and hence able to survive; and, at the lower end of the

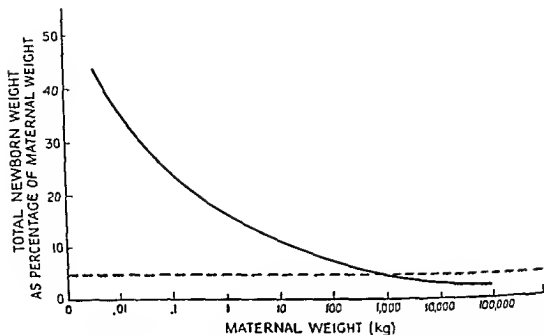


FIG. 2. The relationship between maternal weight and total newborn litter weight (adapted from Leitch *et al.*, 40) together with a 5% individual birth weight (broken line).

distribution by the ability of the fetus to grow sufficiently slowly for it to be less than around 10% of maternal weight, and hence not to suffer from difficulties at parturition. With large strains giving birth to relatively small young, and small strains to relatively large ones, there would be spare "conceptus capacity" in the large strains, but inadequate capacity in the small ones, so that natural selection would favor the birth of numerically larger litters by the heavier strains and vice versa. These facets of mammalian reproduction may be summarized as follows:

$$\text{Log litter weight} = 0.8 \log \text{maternal weight} - 0.33$$

$$\text{Individual birth weight} = 5\% \text{ of maternal weight}$$

Gestation is more closely related to body weight between species than within species

There are many exceptions to these generalizations. For example, bats produce single young of up to  $\frac{1}{3}$  of maternal weight, and at the other extreme, neonatal polar bears are only  $\frac{1}{300}$  maternal body weight. The domestic pig produces more and smaller young than might be expected. Both the generalities and the exceptions are important. The generalities provide some understanding of the processes. They do enable deviations to be detected and measured so that new features may be recognized and explanations sought, and unusual capabilities may be utilized.

Given that such a reproductive system has evolved, it is reasonable to conclude that it is one of the factors which determines both the range of

body weight in mammals, and the association between body weight and litter size within and between species. It is not reasonable to say that these restrictions cannot be broken. The present reproductive system has evolved and is still evolving; the presence of variation demonstrates the possibility of further changes. The relationships outlined, however, help to summarize the extent of genetic variation in reproductive performance, and indicate that the long-term development of new domestic animals may be dependent upon the consideration of these factors rather than simply looking at single characters such as growth rate or litter size. In the meantime, however, the remainder of this chapter will be concerned with the improvement of our present animals, rather than with the esoteric excitement of producing new ones, or of modifying sexual differentiation.

#### IV. Variation within Species

The earlier part of this chapter has dealt primarily with the determination of sex and with differences between species, which are obviously genetic. Variation within species may also be genetic, and may take the form of differences between breeds or strains, or differences between individuals within populations.

##### A. MEASUREMENT OF GENETIC VARIATION

If two groups of animals differ when reared in the same environment and if this difference is present in the next generation, we can conclude that the difference is probably genetic. It is the transmission of the difference to the next generation which is critical, and it is this transmission and element of permanence which is the real value of genetic improvement. Sometimes the resemblance between parent and offspring or between sibs may be striking, as in the case of traits inherited as "single genes." More often, the resemblance will be quantitative rather than qualitative; related animals will tend to be more alike than unrelated animals, and when many genes are involved extensive statistical analysis may be necessary for their demonstration. The measurement of genetic variation is the science of quantitative genetics; a good reference on the subject is the book by Falconer (14).

##### 1. Genetic Selection

Given that a trait is influenced genetically, the object of genetic selection is to increase the frequency of genes with a favorable influence on the

trait in question. Operationally, selection is the choice of parents, and at its extreme this may be preferring one species to another, or the choice of a particular breed within the more suitable species. Improvement within a breed or cross, however, is dependent upon the ability to identify individuals or breeding groups carrying favorable genes to pass on to the next generation, and so increase their frequency. The success of this procedure depends on how much of the superiority of a particular breeding group is transmitted to its offspring. This portion is called the *heritability* of the trait and can be used to indicate the rate of response to genetic selection. The response ( $R$ ) is equal to the superiority of the parents, termed the selection differential ( $S$ )  $\times$  heritability ( $h^2$ ), i.e.,  $R = Sh^2$ . The selection differential may be considered in terms of the proportion of individuals selected, for if the variation in the trait in question is normally distributed, and this assumption is basic to the present discussion, the deviation of an extreme group from the overall mean is a function of the proportion of individuals selected as the elite group and the phenotypic standard deviation of the population, so that  $R = i\sigma_p/i^2$ , where  $i$ , the selection intensity is the number of standard deviations by which the mean of the selected group exceeds that of the overall population and  $\sigma_p$ , the phenotypic standard deviation. As the proportion of the population selected decreases, the superiority of the selected animals increases, but meets with the law of diminishing returns (see tabulation below):

Proportion selected	Selection intensity
0.4	1.0
0.2	1.4
0.1	1.75
0.05	2.06

When the number of selected individuals is fixed, (and this is often the case in practice) it can be seen that a fourfold increase in the size of the population as selection increases from 1 in 5 to 1 in 20 only gives a 50% increase in the rate of response.

The use of standardized units allows different selection intensities on males and females to be pooled, e.g., if 1 in 10 males and 4 in 10 females are selected each generation,  $i$  per generation is  $(1.75 + 1.0)/2$ , and, as  $i$  is a property of the breeding structure of a population, the relative merits of different population structures are easily compared. It also emphasizes the importance of total variation to the rate of response to selection. Response to selection for a trait with a low heritability but high variance could well be greater than that for a trait with low variance no matter how high its heritability.

In terms of animal improvement, the response per year is

$$R \text{ (per year)} = (i/L) \sigma_p h^2$$

when  $L$  is the mean generation interval. The response to selection is, therefore, partly dependent upon the breeding structure of the population which determines  $i/L$ , the annual selection intensity, and partly on the characteristics of the trait in question.

The heritability concept enables us to predict from the present to the future and, like all predictions, it should be used with caution, for it assumes, among other things, that the population parameters do not change! As gene frequencies change as a result of selection, so too might the heritability. Furthermore, it is a statistical abstraction which does not consider specific gene interactions, nor is it sensitive to the effects of single genes with major effects or able to cope with changes in the environment. It is certainly not an end in itself, but provides a framework for the consideration of genetic improvement schemes. It is only as good as the trait in question, and the later sections of the chapter show the importance of understanding the trait to be improved. The simpleminded application of statistical techniques to complex traits is obsolescent.

## 2. Rate of Response to Selection

In a population of a given size, selection among males is limited by the number of females to which a male can be mated, and by the inbreeding which can be tolerated. If selection is too intense leading to the use of too few males, inbreeding may occur, and this is, in general, deleterious. With selection among females, however, the situation is very different, for with sheep and cattle, in particular, the reproductive rate is so low that most of the individuals have to be kept to maintain the population. Selection may only be possible if each individual is mated several times, but this lengthens the generation interval. The reproductive rate of females, and to a lesser extent, the number of females impregnated by a male therefore influence the rate of response to selection.

Selection for reproduction is further complicated by the fact that it can only be measured in the sex concerned—fertility is sex limited. Males either have to be chosen at random or on the basis of the performance of their female relatives reducing the rate of genetic change from selection to half, or less, than that possible if males also expressed the trait.

a. INDIRECT SELECTION. Under certain circumstances it may be more efficient to improve a trait by selecting on one correlated with it, and the possibility of using ovulation rate, for example, or measures of endocrine activity as criteria for selection for litter size will be discussed later. The use of such traits may enable superior individuals to be recognized at an

earlier age and so reduce the generation interval. The success of such a scheme depends upon the annual selection intensity which can be applied to the new trait, and on the genetic relationship between that trait and the trait of commercial importance. With equal selection intensities, the response to indirect selection for trait A via selection of trait B is equal to direct selection on A when  $r_A h_B = h_A$  (where  $r_A$  is the genetic correlation between traits A and B). In order to benefit, therefore,  $h_B$  or the selection intensity on trait B must be greater than for trait A. If A is sex limited, indirect measures on both sexes would be advantageous when  $r_A h_B$  was greater than  $\frac{1}{2} h_A$ , i.e., where  $r_A$  was  $\frac{1}{2}$  or more with equal heritabilities and annual selection intensity.

**b. REPEATED MEASUREMENTS.** The object of selection is to improve the genotype. The heritability of the trait indicates the accuracy with which we can identify superior genotypes in a random breeding population through measures of the phenotype. Indirect selection is beneficial if the genotype of the trait in question can be measured more accurately through the phenotype of an associated trait. The use of repeated measures of a trait is an attempt to reduce environmental variance, and hence increase the accuracy of estimation of the desired genotype. The heritability of a series of  $n$  observations ( $h_n^2$ ) is equal to:

$$h_n^2 = \frac{n}{1 + (n-1)r} h^2$$

where  $r$  is the repeatability of the trait (this includes nongenetic as well as genetic sources of similarity, so that it is the upper limit to the heritability). As the number of observations increases,  $h_n^2$  increases towards  $h^2/r$ . The lower the repeatability, the greater the advantage of taking repeated observations.

The use of correlated traits or repeated measurements may be summarized as attempts to improve the rate of response to selection through increases in  $r_A h$  ( $r_A = 1$  when selecting directly), and are therefore complementary to the effects of changes in annual selection intensity discussed earlier in this section. The response to selection is covered further by Pirchner (52) and Turner and Young (64).

## B. EXISTING VARIATION

### 1. The Effects of "Single Genes"

Single genes in this context cover genetic effects which are inherited as a unit to produce a readily recognizable characteristic or syndrome. The

inheritance of sex is an extreme example of the effects of a single gene. Most single genes are recognized through major changes in reproductivity, and as such tend to be abnormalities; but few abnormalities arise from the effects of single genes. The review of Bishop (5) together with that of Young (70), and the catalog of cattle abnormalities (Lauvergne, 37) cover the subject well.

In males, cryptorchidism and gonadal hypoplasia may both be mainly controlled by single genes. Possibly the most interesting aspect of gonadal hypoplasia is that in some breeds of cattle it reduces the size of the gonads of both sexes, indicating a common autosomal route of control of testicular and ovarian function. Among females, another genetic disease is white heifer disease, where the development of the anterior vagina and cervix is restricted to a varying degree, so that fluid secretions accumulate in the uterus. Selection for muscularity in some French breeds of cattle has led to increases in the incidence of the "culard" gene, which may be associated with reduced fertility and calving difficulties (65).

The elimination of single gene defects is complicated by the fact that many such genes are recessive. The incidence of the defect is then the square of the gene frequency (the proportion of loci in the population represented by the particular gene). At intermediate frequencies, selection is successful, but as the frequency of the gene is reduced, the incidence of the syndrome decreases rapidly. So too does the proportion of the defective genes carried by affected individuals, for this is equal to the gene frequency. When the frequency is reduced to 0.01, for example, only 1 animal in 10,000 is affected but 98 are carriers. Hence, at low frequencies the elimination of affected animals has a trivial effect on the frequency of the gene or the subsequent incidence of the abnormality.

Despite the infertility induced by these genetic abnormalities, and by those concerned with the determination of sex, it can then be seen that they will only be reduced in frequency very slowly, if at all, with natural selection. Their frequency is much more likely to be influenced by artificial selection on dominant genes which are closely linked to them. This is thought to have happened in the case of gonadal hypoplasia, when the linked dominant white coat color gene was favored, and an increase in the frequency of sex reversal in goats may be due to linkage with the polled gene. If it were considered desirable to pursue the elimination of a deleterious gene, breeding trials would be necessary, for example, by crossing with known heterozygotes; however, this is done rarely by artificial insemination (AI) organizations.

To the extent that sections of chromosomes behave as single genes at meiosis, it is appropriate to consider them here. The majority of translocations reported in domestic animals are Robertsonian (i.e., the fusion

of two telocentric chromosomes). Despite the formation of unbalanced gametes there appears to be no clear effect on male fertility in domestic animals (8).

## 2. Breed Differences and Selection Studies

Most of the evidence for the presence of genetic variation in the reproductive performance of domestic animals comes from breed comparisons, and current studies are reported in the Proceedings of the Working Symposium on Breed Evaluation and Crossing Experiments with Farm Animals at Zeist (42). Information was also presented at the "World Congress on Genetics Applied to Livestock Production," Madrid, 1974. Attempts have also been made to improve the reproductive performance of sheep and pigs by genetic selection, and the relationships between relatives have been analyzed in these species and in cattle. These comparisons, analyses, and selection programs are discussed for cattle, sheep, and pigs. The subject of breed comparisons introduces the question of cross breeding and heterosis. Heterosis, defined as the deviation of the cross bred animal from the parental mean, is evidence of the presence of gene interactions. Usually, however, heterosis has to be such that the cross bred exceeds the better parent before it is of practical value but when many components lead to overall profit, the cross may be superior to the better parent, even though each component is not. The reverse of heterosis, inbreeding depression, is also relevant, for again this indicates the presence of nonadditive genetic variation.

a. CATTLE. Breed comparisons are complicated by the deficiency of studies where several breeds have been kept in the same environment, so that the breed differences reported by Ortavant and Thibault (50) could be partly genetic and partly environmental in origin; some genetic variation may nevertheless be inferred. Cundiff (10) reports heterosis of 3 to 6% for conception rate, calves born per 100 cows, and the interval between calving and the next estrus. Calving difficulties were discussed by several authors at Zeist.

Genetic studies of twinning within breeds have been reviewed (1, 18, 23, 55). Both the heritability and repeatability appear to be very low, possibly 4 and 6%, respectively, indicating that the maximum rate of response to selection for twinning in a closed herd would be of the order of 0.1 to 0.15% per year (35), which seems trivial. There are, however, few reports of selection for twinning following initial selection of extreme animals from the national herd, although this has (11) or is being tried by B. M. Bindon and L. R. Piper in Australia and by F. Ménéssier in France.



b. SHEEP. Reproductive performance is closely related to commercial merit in the sheep, and this is reflected in the attention given to different aspects of reproductive performance in the report on breed evaluation and cross-breeding by Maijala (41). The most extreme variation referred to is the litter size of 1.0 for 2- to 4-year-old Tasmanian Merino ewes compared with 2.7 for the Finnish Landrace, with ovulation rates of 1.06 and 2.96, respectively (36). Land *et al.* (36) and Wiener (69) discuss the difficulty of interpreting the wide variation in heterosis reported in the literature, for caution must be paid to the observation of heterosis when one of the parent breeds fails in a particular environment, and it was concluded that heterosis must be measured in the environment to be used. Inbreeding depresses the reproductive performance of sheep; for example, the litter size (No. born/female mated) declined from around 1.60 to 0.97 as the coefficient of inbreeding of the dam increased from 0 to 50% (Wiener, G., personal communication). Additive and nonadditive genetic variation affect litter size, ovulation rate, and age at maturity.

Bradford (6) records in his comprehensive review of the genetic control of reproduction in sheep that genetic variation within breeds has been observed by sib analyses, and that attempts have been made to exploit this by selection. Wallace (67) and Turner (63) have each reported progress, of the order of 0.025 lambs per ewe per year, and Bradford himself reports early success in selection. He concludes, however, that although "the results obtained to date demonstrate that litter size can be increased by selection, the experiments have not been designed to provide quantitative estimates of realized heritability, nor is it probable that initial rates of response will be maintained over a longer period of time." The values for the heritability of litter size referred to indicate that it may be of the order of 0.1.

c. PIGS. King (27), in his review of papers presented at Zeist says "the results confirm the generally beneficial effects of cross breeding and showed that the large benefits derived from cross breeding females must have been due to an improvement of embryonic viability rather than ovulation rate." In addition, the "different amounts of heterosis observed in different breed crosses illustrate the shortcomings of assuming average amounts of heterosis in untested breed comparisons." The differences to be expected between breeds are illustrated by the comparison of 28 crosses among 8 breeds (20) and by Omtvedt's (49) summary of swine breeding in the United States. He concludes from the Oklahoma crossing project that 7% more corpora lutea were represented as embryos when purebred gilts were crossed rather than mated to boars of their own breed, and that litter size and litter weight at 42 days were raised by 18 and 21%, respec-

tively. The use of cross bred gilts raised these increases to 16, 29, and 32%, respectively. [These references together with the comprehensive review by Sellier (57) serve as a base to the extensive literature on the pig.]

Breed differences therefore illustrate the presence of genetic variation in litter size and its components, ovulation rate, and embryo survival. The effects of inbreeding on reproductive performance when the litter size of Large White gilts was reduced from 10.2 to 7.6 as inbreeding increased to 40% (26) indicate the presence of nonadditive genetic variation.

Within the Large White breed, the heritability and repeatability of the number born have been reported to be 7 and 15%, respectively (60). Reproductive performance was not significantly genetically correlated with other performance traits, so that genetic selection for one would be expected to leave the others unaffected (45). Selection within the same breed led to changes in litter size with a realized heritability of  $0.25 \pm 0.37$  (48).

It has been suggested that the heritability of litter size could be underestimated by dam-daughter regressions owing to the presence of a maternal effect where the litter size of individuals born in large litters is reduced environmentally and vice versa (53) and higher, but not significantly higher, heritabilities based on granddam-granddaughter than dam-daughter regressions were reported. If this were the case one would also expect half sib heritability estimates to be greater than parent offspring ones, but the evidence for this in the literature is not conclusive.

### 3. Genotype by Environment Interactions

The sensitivity of reproduction to environmental stimuli such as day-length and nutrition is well-documented in some species. When two genotypes react differently to a change in the environment there is said to be a genotype by environment ( $G \times E$ ) interaction. Possibly the best examples of this are the response of sheep to changes in nutrition, and effects of tropical stress on the fertility of cattle.

Doney and Gunn (13) report that the ovulation rate of Blackface sheep changes from 1.3 to 2.0 as the body condition score changes from 3 to 5. A similar change in body condition, however, leaves the ovulation rate of the Finn  $\times$  Blackface and South Country Cheviot sheep relatively constant around 2.7 and 1.3, respectively. The ability of sheep such as the Blackface to adapt to their environment may be very important.

The change from a temperate to a tropical climate reduces the fertility of temperate breeds of cattle (51) and crosses with tropical breeds show considerable heterosis (56) but this may arise from the failure of one of the parental breeds in a particular environment. Indeed, in view of the

sensitivity of reproductive traits to environmental stimuli, it is perhaps surprising that crosses should ever be exactly intermediate between their parents, for this is dependent on both genetic additivity and the equal suitability of the environment for all three genotypes. The environment has many components, the response to each of which may be inherited additively, but if the effects of the environment on the genotype are not linear, the resultant cross bred superiority would be spuriously measured as nonadditive genetic variation. Breeding programs designed to exploit nonadditive variation should consider this.

The response of sheep to changes in the photoperiodic and nutritional components of the environment are discussed in Chapters 18 and 21. A clear example of the various reactions of different breeds of sheep to the same environment is given by the recent study of Tasmanian Merino, Scottish Blackface, and Finnish Landrace females, all kept in the same conditions in the Northern Hemisphere (68). The Merinos showed estrus first, around the beginning of September, followed by both the Blackfaces and Finns in early October. At the other end of the season, the Merinos and Blackfaces both finished around the end of February, whereas the Finns continued to show heat until the end of April. The breeding season of the Blackfaces was therefore symmetrical about the winter solstice, the Merino season was displaced forward, and the Finn season backward. The genotype of the sheep not only determines that it responds to changes in daylength, but also the way in which it responds. The behavior of Finn and Blackface rams has also been monitored in the same environment, and the variation was found to follow a similar pattern to that of the females, indicating that seasonal variation is controlled in part by the same genes in both sexes (29). The extent to which reproductive traits may be expressed in both sexes will be pursued later.

### C. IMPROVEMENT OF REPRODUCTIVE PERFORMANCE

Reproductive performance may be divided into two components, the number of eggs shed by the ovary, and the ability of these eggs to develop to parturition as live young. Many authors have considered raising the prolificacy of domestic animals by selecting for ovulation rate. The extent to which litter size is limited by the ovulation rate may be measured as the correlation between the two variables, and from the point of view of genetic change, the genetic correlation. As we saw earlier, for selection on ovulation rate to be beneficial, either the intensity of selection must be greater, or  $h_{ov}$  must be greater than  $h_L$  ( $h_{ov}^2$  and  $h_L^2$  = heritability of ovulation rate and litter size, respectively).

Several workers have attempted to divide variation in ovulation rate

into that arising from gonadotropic stimulation and that arising from ovarian sensitivity. The techniques used, however, have been restricted to assessing the response of the ovaries to a series of doses of exogenous gonadotropin, usually PMSG. A fundamental deficiency in this approach is that it is not possible to separate the response of the ovary at a given time from the effects of exposure to endogenous gonadotropins up to that time. The difficulty is illustrated by the observation that the number of oocytes in the ovaries of newborn lambs is negatively correlated with the subsequent probability of twinning (28, 62). This relationship was postulated to be associated with differences in gonadotropic stimulation, and is supported by the results of studies in laboratory animals (see Chapter 7). The response to PMSG cannot be considered to have a common starting point. Elucidation of this problem awaits more critical tests, for example, the transfer of early embryonic ovaries between strains.

## 1. Ovulation Rate

Genetic variation in ovulation rate is present in sheep, and Bradford (6) concludes from the results of embryo transfer studies, superovulation, and the comparison of different breeds that this is the main limit to present day reproductive performance.

The heritability of ovulation rate has not been estimated in sheep, nor has the genetic correlation with litter size. Hanrahan (16) bases his discussion of selection for ovulation rate on a repeatability estimate of 0.1. This, however, seems either to be an underestimate, or to be specific to the Galway sheep and he has since communicated higher values for other breed types. Further there are estimates of 0.9 in Merino sheep (3) and of 0.3 in the Ile de France (Thimonier, J., personal communication). Hanrahan nevertheless concludes that selection could be based on ovulation even within Galways largely because of the reduced generation interval.

In pigs, the breed comparisons discussed earlier report the presence of genetic variation, and Zimmerman and Cunningham (72) report considerable success in selecting for ovulation rate in a crossbred population, with a heritability of around 0.5, and a superiority of the selected line over the control of 2.5 corpora lutea after five generations. Legault and Ollivier (39), however, quote 0.25 as the heritability of ovulation rate within a breed, and a genetic correlation of 0.25 between it and litter size, so that  $h_{0r_A}$  would be equal to 0.12, i.e., less than  $h_L$  if  $h_L^2 = 0.07$ . The critical unknowns, therefore, are the possibility of raising  $h_0^2$  by the use of repeat measurements and the change in litter size achieved by Zimmerman and Cunningham.

There are no estimates of genetic variation in ovulation rate within

cattle populations but in view of the extreme difficulty in selecting for twinning itself, Land and Hill (35) considered the identification of superior cows by repeated laparoscopy, followed by their superovulation and the transfer of their embryos to other cows. The observation of 13% multiple ovulations with only 1.9% twinning (25) suggests caution, but multiple ovulations were diagnosed by rectal palpation rather than direct observation.

Where they are available, estimates of the heritability of ovulation rate are greater than those of litter size, and an increase of twofold would be a conservative estimate. The main advantage of selecting on ovulation rate is, however, that repeat measurements can be made over a short period of time, and hence without lengthening the generation interval. Jacques Thimonier may have a greater impact on the genetic improvement of reproductive performance than he realized when he pioneered the use of laparoscopy in farm animals.

The use of repeated measures of ovulation rate depends upon the genetic parameters of the population concerned. In particular, it depends upon the extent to which ovulation rate limits litter size, for this is the basis of the genetic correlation between the two variables. At one extreme, if the proportion of embryos carried to term was independent of the number of eggs shed, all the genetic variation in litter size would be that in ovulation rate, and hence the genetic correlation between the two traits would be one. Increases in ovulation rate with unchanged and finite uterine capacity will lead to the reverse situation where there is always an excess number of eggs, so that the genetic correlation would gradually decline to nought.

Rather than measure the genetic correlation in a particular population, many workers have attempted to answer the question "would it be useful to improve litter size through improvements in ovulation rate?" by transferring larger than normal numbers of embryos to recipient females.

## 2. Embryo Survival

In sheep, Lawson and Rowson (38) were the first to suggest that the uterine capacity may differ among breeds, and a similar indication was observed by Bradford *et al.* (7). These indicative studies were based on the transfer of relative small numbers of embryos; the superiority of the Finnish ewe over the Scottish Blackface was shown more conclusively by the transfer of fourteen eggs per ewe, when the survival rate was 2.5-fold greater in the former (Land, R. B., and Wilmot, I., unpublished). At this number of embryos, survival had declined to 10 and 4% in the Finn and Blackface ewes, respectively.

The declining proportion of eggs surviving as the ovulation rate increases, together with the initial low survival rate in cattle (estimated from nonreturn data to be 60% or less), indicates that the ovulation of three to four or possibly more eggs if the decline is steeper in cattle than sheep would be required to ensure an average birth rate of two calves. The probability of all of four ovulations occurring on the same ovary, assuming independence, would be 1 in 8 ( $2 \times 0.5^4$ ), so that the possibility of lower fertility with unilateral pregnancies (54) may not be a real problem.

Any proposal to improve the reproductive performance of domestic animals genetically should, therefore, consider the use of ovulation rate as a selection criterion. This is more likely to be useful in cattle and sheep than in pigs, where both the counting of corpora lutea by laparoscopy may be more difficult and where the genetic correlation between ovulation rate and litter size may be lower (71).

Where improvements in ovulation rate are desirable, the possibility of crossing as well as selection should be considered. The development of breeds with extreme ovulation rates may be beneficial; "commercial breeds" could be improved with a minimum dilution of their other advantages.

## D. PHYSIOLOGICAL AIDS TO SELECTION

### 1. Quantitative Physiology

Given that it may be desirable to improve the ovulation rate of domestic animals, there are many practical disadvantages. For example, it does not lend itself to "on farm" evaluation. Several studies have therefore been made of endocrinological variation associated with differences in ovulation rate (32, 33). The significant observations were that Romanov ewes with an ovulation rate of about three had a longer interval between the onset of estrus and the release of LH although no differences were observed in the concentration of LH during the rest of the estrous cycle, and that the concentration of LH in the plasma of lambs of both sexes was higher in breeds and crosses with high ovulation rates than in those with low ovulation rates. The differences in LH concentration cover a relatively limited period (4) and the correlation between successive ages (Fig. 3) was only 0.2 (Blanc *et al.*, personal communication). The Ile de France and Préalpes lambs may go through a similar peak in LH concentration to that of the Romanovs, but at a later age. The lamb results support the suggestion that genetic differences in reproductive activity are expressed in both sexes, and hence that it may be possible to make direct estimates on males of the reproductive activity of their potential daughters (30).

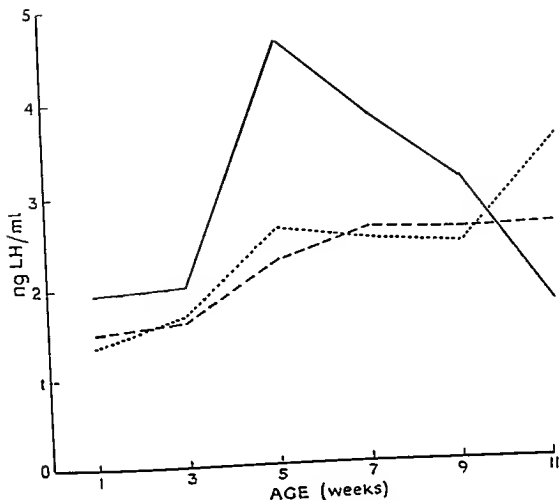


FIG 3 The mean concentration of LH in the plasma of ram lambs of differing prolificacy from 1 to 11 weeks of age. Solid line Romanov ( $n = 20$ ), dashed line Ile de France ( $n = 92$ ); dotted line Préalpes ( $n = 40$ ) (From Blanc *et al.*, personal communication)

The comparison of breed types with high and low ovulation rates has been pursued, and possibly the most significant observation has been that the growth rate of the testes of ram lambs is related to the reproductive performance of females of their breed type. This was first observed in a comparison of Finn and Merino ram lambs (31) and subsequently confirmed with other breeds (9, 17). The common factor underlying the high reproductive activity of males and females may be reduced hypothalamic sensitivity to negative feedback from gonadal steroids (34). This would explain the earlier failure to observe differences in LH concentration during the estrous cycle.

Luteinizing hormone, of course, is not the only gonadotropin concerned with the physiology of ovarian activity, and it was perhaps fortunate that LH was both the gonadotropin whose assay in plasma was developed first and one which showed a quantitative relationship with female reproductive activity. Variation in other hormones may also be found to be related

TABLE I

*The Concentration of FSH (ng/ml) In the Plasma of 30-Day-Old Male and Female Merino Lambs of Strains Selected for Twinning\**

Strain	Male	Female
Control (C)	17.8	42.0
Low twinning (O)	20.0	25.1
High twinning (T)	17.2	92.5
High twinning (B)	21.2	124.7

\* From Bindon and Allison (personal communication).

to the end product of reproductive activity. Indeed, some of the early results of Bindon and Allison (personal communication) from their study of strains of Merino selected for and against twinning show that the concentration of FSH in the ewe lamb may be related to the frequency of twinning of the strain (Table I). However, the differences were not found in males of the ages studied.

It is therefore possible to conclude that in the sheep reproductive activity is a trait common to both sexes, and it is only the nature of this activity that is sex limited. This brings us back to earlier sections of the chapter; the gonadal hypoplasia syndrome was expressed in both sexes, and the conclusion of the discussion of sex determination was that although sexual differentiation may be triggered by the Y chromosome, the expression of sex is controlled by the autosomes or the X chromosome.

These studies illustrate the presence of a quantitative relationship between the hormones of the physiological pathways underlying reproductive performance and the level of performance obtained. Although it is still in its infancy, the quantitative physiological comparison of genetic differences is helping to reveal which of the physiological steps leading to the production of young are the critical ones and to indicate where the quantitative control may lie. The observation that genetic variation in litter size may be associated with steroidal feedback on the hypothalamus (34), for example, indicates that it may be possible to raise litter size by modifying this feedback. This would have the advantage that normal buffering systems would still operate, thus avoiding the large increase in variation which follows the direct stimulation of the ovaries with exogenous gonadotropins. The use of normal variation of quantitative genetic origin may well add to the classic physiological techniques of ablation, supplementation, and immunization to induce variation artificially. This approach therefore helps the physiologist to understand which traits are important, and to consider where physiological intervention may be most appropriate. In the



next section we shall see how it might help the geneticist to improve his selection criteria.

## 2. Selection Criteria

The concentration of LH in the plasma of young animals could be used as an indicator of potential breeding value, but this is very variable in the lamb. This drawback might be overcome by using the response to releasing hormones, but early studies in selected Merino strains (Bindon, B. M., personal communication) and British breeds of sheep (Carr *et al.* personal communication) gave conflicting results. Should breed or strain differences prove to be associated with female performance and if the relationship holds within populations, the concentration of LH at a set time after the injection of releasing hormone could be readily used in the field.

Alternatively, the association of testis size with female performance may provide the key to a very simple form of selection. This has been tried in mice (22) and sheep (33). In both, the heritability of testis size was found to be around 0.5. Selection of rams on testis growth during a decreasing (i.e., inhibitory) photoperiod led to changes in the onset of the breeding season of their daughters. Replicated selection among mice has led to correlated responses in the ovulation rate of the females—solely through selection on the weight of the testes of males and independent of body weight. The genetic correlation between the two traits was around 0.5.

The incorporation of such a male criterion into a selection program for female performance would give an  $r_{fh}$  of around 0.35, assuming that ovulation rate limits litter size. In comparison, using an estimate of 0.1 for the heritability of litter size,  $h_o$  would be 0.3 and  $1/2h_o$  0.15, so the response in litter size to selection among males on their own performance would be around twice as great as when selection was based on their mothers' performance. Further work, is required to determine the most suitable male criteria, which will be a compromise between maximizing  $r_{fh}$  and minimizing the effort and cost of measuring the trait (even keeping males uncastrated, for example, may constitute an expense).

The potential use of male characteristics is not limited to their association with "female puberty" and litter size, but the observation of breed differences in seasonal variation in testis diameter of rams indicates that it may be related to seasonal variation in ewe fertility (21). The male characters considered may be related to ovarian activity rather than litter size, so that as in the case of selection for ovulation rate, success could reduce the genetic correlation between them and litter size, and hence reduce their effectiveness.

### 3. Artificial Changes in Reproductive Performance

We have already seen that the reproductive performance of males is usually so great that it does not limit selection within a population. The use of artificial insemination however, enables superior genetic material to be disseminated more rapidly, and also facilitates progeny testing of males on a large scale. Most national dairy improvement schemes are based on the contemporary comparison of the daughters of different bulls within the same herds, a scheme which would be unacceptable without AI.

As has been shown earlier, the situation is quite different in females, where, especially in sheep and cattle, selection is limited by the large proportion needed as replacements. The possibility of increasing selection among females through the use of superovulation and embryo transfer to increase their rate of reproduction has therefore been studied. The main point to recognize, however, is that the best that can be achieved by increasing the rate of female reproduction is to raise  $i/L$  in females to that which is already at an optimum for males. No matter how great the increase in female reproductive performance, it can no more than double the rate of response to selection. Detailed studies (35) show that the rate of response to selection for body weight is increased by around 60% by increasing calf yields to four to ten per female (Fig. 4). The insensitivity of the increase to changes in calf yield can also be seen.

The progeny testing of dairy bulls through AI tempts one to suggest that dairy cows with good records could be progeny tested by embryo transfer, but the genetic advantages are marginal, and the effort would not appear to be worthwhile (19). Again, in contrast to the benefits of AI, the use of embryo transfer to disseminate genetic merit is likely to be limited at present, partly because of inadequate sources of embryos.

**SELECTION MARKERS.** A final comment on indirect selection must be given to the technique of randomly choosing easily measurable variables such as blood groups and looking for possible correlations with reproductive characteristics. Although there are some examples of the successful application of this technique, it is illogical and may be compared with techniques of, for example, using horn shape or coat color to select suitable animals.

### E. PRACTICAL IMPROVEMENT SCHEMES

There is genetic variation in reproductive performance; should it be exploited in genetic improvement schemes?

In cattle, the slow rates of response indicate that selection for fertility at the expense of selection for milk yield in dairy breeds may lead to the

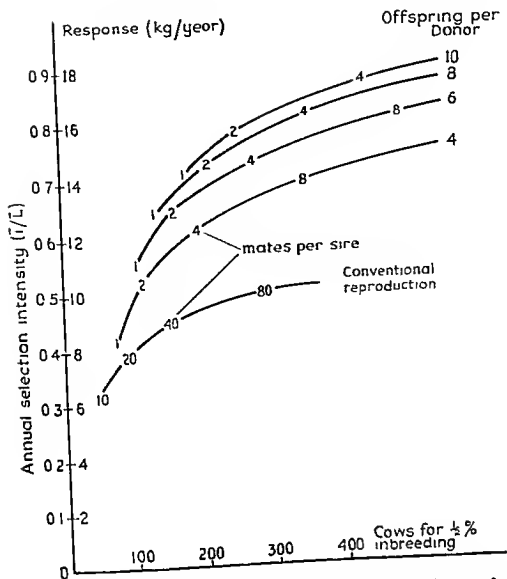


FIG 4. The comparison of conventional schemes of cattle selection for traits of the growing animal with those incorporating superovulation and embryo transfer to raise female reproductive rate. Response is shown in relative terms as the annual selection intensities, and as the predicted response in 400 day weight if  $h^2 = 0.5$  and  $\sigma = 40$  kg, for different population sizes represented as the number of mates per bull and as the total number of cows (donors and recipients) for  $\frac{1}{2}\%$  inbreeding per year. (From Tables 1 and 2, Land and Hill, 35)

formation of a population which could not compete overall with those that had concentrated on the improvement of milk yield, even though there may be a small positive correlation between milk yield and fertility. In contrast, dam overheads contribute such a large proportion of the costs of lamb production, that selection for reproductivity would be advantageous. These points summarize the factors which have to be taken into consideration when assessing the merits of incorporating selection for reproductive performance into genetic improvement schemes: the economic responses to selection for the various traits and the genetic correlations between the traits. The best way to combine traits for selection is to use a selection index, although under some circumstances the use of inde-

pendent culling levels may be as good (64), but the details are outside the scope of this chapter, and the balance between selection for reproductive and productive traits can be seen from the profit contour technique (44). In the pig, for example, selection for litter size, for food conversion efficiency, or for a combination of the two is considered in terms of the amount of selection (*i*) required to give the same change in profit. As one of the two components of profit improves, the selection required per unit of further improvement increases, so that the optimum balance moves in favor of the other. The costs of achieving a particular intensity of selection were not included, and this could affect the balance in two ways. On the one hand, as reproduction is sex limited the population required for a given selection intensity for number born would be larger than that for food conversion ratio. On the other hand, the measurement of food conversion efficiency is expensive, whereas that of reproduction is inexpensive, so that for any given litter size the merits of selection for litter size increase as the cost of recording food conversion increases (39). The balance can be further modified with the development of specialized sire and dam lines.

The optimum procedure therefore changes even assuming that the genetic characteristics of the population remain constant, and constancy of genetic characteristics is the basic assumption in all the genetic calculations discussed earlier. As selection proceeds, however, the gene frequencies change, the additive genetic variance for the trait in question declines, and the response per unit of selection declines, leading eventually to plateaued populations. Superimposed upon the economic considerations, is the genetic consideration that as the limit to selection is reached, the relative genetic effort required to achieve a particular change also increases. The qualifications to the use of the predictive response equations are very real.

To return to the original comment that at present it is better to select for milk yield than twinning in dairy cows, this situation would change if the relative economic weightings of milk versus beef were to change, or if, as selection for milk yield progresses, the heritability of twinning were to increase relative to that of milk yield. The current disagreement over the optimum size of beef cattle however indicates that in the absence of a clear size objective, selection could be applied usefully to twinning in beef cattle. In pigs the present commercial breeding systems may be summarized as the use of crossing to exploit hybrid vigor in the litter size of dams, and selection to increase the growth rate of sire lines. The pig industry could however reach the stage of development when the response to selection for growth traits declines so that selection for reproduction may become profitable. Commercial within breed selection of sheep is

not yet established, but crossing schemes, using the additive variation in litter size, and the exploitation of genotype  $\times$  environment interactions are normal practice.

## V. Synthesis

Genetics is both a part of reproduction and a means of improving reproduction. The genetic control of reproductive performance is not in doubt. In comparison to performance traits, however, a lower proportion of variation in reproductive traits within populations is genetic in origin, leading to lower heritabilities for the latter. It must be remembered however that reproductive traits are not necessarily under less genetic control. On the contrary, the restricted variation within populations indicates that the genetic control is stricter, and it may be useful to think of reproductive traits as having a higher degree of genetic determination. Natural selection may have reduced the segregation of genes which influence traits important in evolution, but not of genes controlling traits which are less closely related to the fitness of the individual. It is also to be remembered that a low heritability does not necessarily mean a slow response to genetic selection; the variance of a trait is also important, and many reproductive traits have high variances.

Within animals, reproductive performance is not a collection of unrelated traits but a central character expressed in several ways. As the earlier sections of this book have shown, traits such as age at puberty, sperm production, and litter size all have common physiological elements. The genetic studies show that these elements are controlled by autosomal genes and hence the genetic variation is not sex limited, though the traits may be; and further that quantitative effects of these genes in different traits leads to an observed correlation between the traits. Reproduction therefore illustrates a situation where the end products of gene action can be measured as a genetic correlation between different traits, and where the physiological pathways effecting the action of the genes are also understood. To argue whether traits are correlated physiologically or genetically, or whether they are limited genetically or physiologically is therefore fruitless—it is a question of the level of a single control system.

The realization that reproductive performance is subject to genetic variation adds to the classic methods of physiology. In addition to the creation of artificial systems by ablation or supplementation, normal variation can be induced by genetic substitution. On the one hand, physiology may elucidate genetic variation, on the other, genetic variation may aid physiological studies.

The improvement of the reproductive performance of domestic animals may be helped by the realization that many components of reproductive performance originate from the same genes, and that the expression of these genes can be measured in both sexes. The advantages to be gained by overcoming the present sex-limited measurement of reproductive performance could have an impact on generation intervals, selection intensities, rates of response to selection, and hence the emphasis given to reproductive performance in future improvement schemes. The study of genetic variation in reproductive performance has led not only to progress in the understanding of physiological pathways but also to the real possibility of using this knowledge to improve the agricultural merit of our domestic animals.

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# 23 Infectious Diseases Influencing Reproduction

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## I. Introduction

This chapter presents a brief account of the major infectious diseases which affect fertility, induce abortion, or cause abnormalities of progeny born at term. These infections are of interest for economic and public health reasons. They may create serious problems for the commercial livestock producer since several of the diseases are chronic and affect herd health over a period of many years. Some of the infections may strike unexpectedly to cause extensive losses in herd productivity, thus creating an added dimension of uncertainty in livestock production. Research workers in the field of reproduction in domestic animals need to be aware of these infections which may affect their data. When hazards to human health

TABLE 1

## Bacterial, Viral, and Protozoan Infections Affecting Reproduction

Disease	Species	Etiology	Reproductive effect	Control
<u>Bacterial infections</u>				
Brucellosis	Cattle	<i>Brucella abortus</i>	Late abortion, sterility in bulls	Immunization, test and slaughter
	Sheep, goats	<i>Brucella melitensis</i>	Abortion	Immunization, test and slaughter
	Swine	<i>Brucella suis</i>	Abortion, weak pigs	Test and slaughter
Vibriosis	Dogs	<i>Brucella canis</i>	Abortion	Sanitation
	Cattle	<i>Campylobacter fetus</i> , subspecies <i>fetus</i>	Infertility, early abortion	Immunization, artificial insemination, chemotherapy
	Sheep	<i>Campylobacter fetus</i> , subspecies <i>intestinalis</i>	Abortion in last trimester	Immunization
Leptospirosis	Cattle	<i>Leptospira pomona</i>	Abortion in last trimester	Immunization, sanitation
	Swine	<i>Leptospira pomona</i>	Late abortion, weak pigs	Immunization, chemotherapy
	Horses	<i>Leptospira pomona</i>	Late abortion	Immunization, chemotherapy
Listeriosis	Cattle, sheep	<i>Listeria monocytogenes</i>	Abortion in latter half of gestation	Chemotherapy, sanitation
Epididymitis in rams	Sheep	<i>Brucella ovis</i>	Infertility in rams, late abortion	Immunization, isolation
<u>Viral infections</u>				
Equine virus abortion	Horses	Equine herpes-virus 1	Abortion in last trimester	Immunization, (controlled infection)
Equine viral arteritis	Horses	Equine arteritis virus	Abortion in latter half of gestation	Immunization (experimental)
Infectious bovine rhinotracheitis or pustular vulvovaginitis	Cattle	IBR-IPV virus	Abortion in latter half of gestation, temporary infertility in cows and bulls, fetal infection	Immunization, cessation of breeding

TABLE I (continued)

Disease	Species	Etiology	Reproductive effect	Control
Bovine viral diarrhea	Cattle	BVD MD virus	Abortion in first trimester, fetal, deformities	Immunization
Epizootic bovine abortion	Cattle	Virus?	Abortion in last trimester	None
Enzootic Abortion of ewes	Sheep	<i>Chlamydia psittaci</i>	Late abortion	Immunization
Bluetongue	Sheep	Modified live virus vaccine	Fetal deformities	Avoid immunization of pregnant ewes
Hog cholera	Swine	Modified live virus vaccine	Fetal deformities	Avoid immunization of pregnant sows
<b>Protozoan infections</b>				
Trichomoniasis	Cattle	<i>Trichomonas foetus</i>	Abortion in first trimester, sterility in cows	Eliminate infected semen, rest from breeding therapy for bulls
Toxoplasmosis	Cattle, sheep, swine, dogs, cats	<i>Toxoplasma gondii</i>	Abortions, still births premature births fetal abnormalities	Prevent ingestion of oocysts from cat feces prevent ingestion of infected meat, chemotherapy

exist, as is the case with some of the infections, additional procedures for animal disease control are required. Some aspects of these disease problems are summarized in Table I.

## II. Bacterial Infections

### A BRUCELLOSIS

#### 1. The Disease

Brucellosis is a worldwide disease problem affecting a variety of mammalian species, including man. Extensive studies have evolved a battery of differential characteristics for the separation of the three principal species of the genus, in addition to biotypes within each species (30). Identification of the *Brucella* species is difficult since the organisms are similar in physical appearance, share common antigens, and have many

physiological characteristics in common. The most practical aspect of speciation within the genus relates to host specificity, wherein the common reservoir hosts are as follows: *Brucella abortus*, cattle; *B. suis*, swine; *B. melitensis*, sheep and goats. All three of these species may produce brucellosis in man.

The organism may enter the body through a variety of routes but ingestion is probably the most common mode of entry. Venereal transmission from bulls with lesions in the epididymis and testicle may also occur. This aspect of transmission has relevance to artificial insemination. Milk from infected cows commonly contains the organism, which may be viable in fresh milk, butter, and cheese.

Brucellosis in a susceptible herd of animals is associated with abortion following a primary attack by the agent. This may affect a very high percentage of the pregnant animals. A small percentage of abortions occur in subsequent years, but a new storm of abortions may follow additions to the herd of susceptible adult cattle or animals in their first pregnancy. Clinical signs of brucellosis are abortion in the latter half of pregnancy, frequent retention of fetal membranes, and sterility that may follow severe metritis in females and orchitis in males.

The organism commonly persists in the udder and lymph nodes of the mammary gland. In many cases the infection continues over several lactations or even for the life of the animal. During pregnancy, the organisms replicate in great numbers in the gravid uterus. Widespread necrosis and exudation occur in the uterine caruncles and the placenta. The chorio-allantoic membrane is extensively involved with the organisms multiplying intracellularly. An explanation for this predilection to the site of fetal attachment has been presented with the demonstration that erythritol, a poly-alcohol, stimulates the growth of brucellae. This growth factor may be found in high yield in the placentas of species prone to experience abortion from *Brucella* infections (70).

Young animals are generally more resistant to infection than sexually mature adults. Thus, a calf born from an infected dam may have *Brucella* organisms in its tissues at the time of birth and may ingest many organisms when nursing, but these do not produce a clinical process in the young animal and, within a month or two, the agent is usually eliminated from the tissues.

## 2. Diagnosis and Control

Programs exist in many countries for the control and eventual eradication of brucellosis in cattle, goats, sheep, and swine. Clinical signs are of limited value in diagnosing brucellosis because of the chronic and insidious nature of the disease. Isolation of the organism offers an undisputed basis

for diagnosis, and attempted isolation is an important aspect of the diagnosis in man and in certain naturally occurring infections in animals. However, isolation is a costly and time-consuming procedure which is not applied in routine disease control.

The principal means of diagnosis is based upon immunological responses of the infected individual. Infected subjects display hypersensitivity of the delayed type and synthesis of humoral antibodies which may be detected by a variety of methods. There are procedures for detecting complement-fixing antibodies, agglutinating antibodies, and nonagglutinating or so-called "incomplete antibodies."

The agglutination test is a good means for detecting antibody response from stimulation by *Brucella* antigens. Most commonly used is the rapid plate agglutination test, wherein a concentrated suspension of stained *Brucella* organisms is mixed on a glass plate with differing volumes of serum or whey. Agglutination occurs within a few minutes when antibodies are present. The procedure is adjusted to correlate with the more classic tube test. For practical reasons, it has been necessary to define agglutination at a certain titer as indicative of infection, making the animal subject to removal from the herd. It is recommended that the minimum diagnostic level be 100 International Units (IU)/ml for nonvaccinated animals and 200 IU/ml for vaccinated animals. The IU is based on an International Standard for Anti-*Brucella abortus* Serum, which may be obtained from the International Laboratory for Biological Standards, Ministry of Agriculture, Fisheries and Food, Central Veterinary Laboratory, Weybridge, Surrey, England. It is sometimes difficult to interpret tests from animals with low titers of antibodies in their body fluids. Supplementary tests may then be used to clarify the status of animals in the "suspect" classification. The principle involved in procedures where Rivanol or 2-mercaptoethanol interact with the serum is inactivation of the IgM class of antibodies, while allowing detection of antibodies from other immunoglobulin classes (30, 49). The complement fixation test and the antiglobulin test (Coombs' test) may be used to detect "incomplete antibodies" (3, 30).

An important adjunct to the control of bovine brucellosis is the milk ring test (MRT) for herd diagnosis (8, p. 206). In this procedure, an aliquot of whole milk, which may have been contributed from several animals, is mixed with a suspension of inactivated *B. abortus* organisms stained with hematoxylin. In the presence of antibodies, the agglutinated masses of bacteria tend to be carried up into the cream layer. The procedure has great value in control programs, since it obviates the necessity for collecting individual blood samples or even individual milk samples.

The control of brucellosis has depended upon the detection of infected individuals to prevent their introduction into susceptible herds, immuniza-

tion to raise the general level of resistance of replacement animals on infected premises, and last, the test and slaughter of infected individuals when the level of infection has been reduced to a point that this becomes economically feasible. Brucellosis is worldwide, and the current stages of the control programs vary in different regions. Control of bovine brucellosis in the United States is now at an advanced stage, with eradication of the disease in many states and a phasing out of immunization programs followed by extensive use of the test and slaughter procedure.

Immunization has proved to be an indispensable facet of brucellosis control in heavily infected areas. Strain 19 of *Brucella abortus* continues to be the most advantageous agent for immunizing against bovine brucellosis. The organisms are alive and produce a mild form of brucellosis, which can render the vaccine dangerous to man and animals unless used properly. It is highly agglutinogenic in adult animals. This characteristic has caused extensive problems in the control programs where efforts are made to separate antibody response to the vaccine from that caused by natural infection. Calftood vaccination has been the best approach to this problem since the agglutinin response of the young bovine is ordinarily a temporary one and diminishes sufficiently to avoid confusion with titers from field infections. Immunization of heifer calves from 3 to 8 months of age is the common practice. Abortions can occur if pregnant animals are vaccinated. An inactivated vaccine known as 45/20 adjuvant vaccine is reported to have value in raising the resistance of animals where "pools of infection" appear in otherwise disease-free areas. While the vaccine is not as immunogenic as strain 19 *B. abortus*, it does have the advantages of being nonviable and nearly nonagglutinogenic (16).

Reliable means for the immunization of swine have not yet been developed. Goats and sheep may be immunized against *B. melitensis* infection with the Rev 1 vaccine which is a living avirulent strain of *B. melitensis*. A formalin-killed preparation identified as H38 adjuvant vaccine is also recommended under some circumstances.

In recent years, another form of brucellosis has come to the attention of investigators. A new species identified as *Brucella canis* causes a form of brucellosis characterized by abortion and persistent bacteremia in dogs. The Beagle breed is particularly susceptible (13).

## B. VIBRIOSIS

### 1. The Disease

The diseases referred to as vibriosis of cattle and sheep are well-established in the literature under that name and will be referred to as such

here. However, the etiological agent has been reclassified as a member of the genus *Campylobacter* rather than *Vibrio* (10). *Campylobacter fetus*, subspecies *fetus* causes abortion and infertility in cattle. It is transmitted venereally and does not multiply in the gastrointestinal tract. *Campylobacter fetus*, subspecies *intestinalis* causes abortion in sheep and may sporadically induce abortion in cattle and man. The agent is transmitted orally and can be isolated from the gastrointestinal tract and the gall bladders of ewes and cows. The subspecies of *Campylobacter fetus* may be differentiated bacteriologically by some subtle biochemical tests and serologically by their differing surface antigens.

Vibriosis has been considered by some investigators as the most important microorganism causing infertility in cattle. It is of greater importance in beef cattle than dairy cattle because of the limited use of natural breeding in the management of dairy herds. The bacterium is disseminated through the herd by venereal means, causing failure to conceive, early embryo death. Both events leave the impression of infertility since only a few of the aborted fetuses may be found. Embryos killed in early stages of development may simply be resorbed. After 2 to 5 months, the infected females recover from the infection and experience a period of increased resistance which eventually wanes and again leaves them susceptible to reinfection. While most of the cows eliminate the microorganism, a smaller percentage experience persistent cervicovaginal infection which renders them asymptomatic carriers that may transmit the infection at coitus (14). Bulls show no clinical sign even though the organism may be a permanent resident of the male genital organs. Recognized abortions are most common in midgestation, and lesions of the placenta are similar to those occurring in brucellosis. The invading bacterium may be widely disseminated in the fetus. Frequently, the organisms can be obtained in pure culture from fetal stomach contents.

In ovine vibriosis it is assumed that the organism is picked up by the susceptible host from feed and water contaminated by the discharges of infected animals. Since foci of infection may persist in the gall bladders of some ewes, shedding of the organism from this site may maintain the infection in the band. Vibriosis of sheep can be a dramatic event. It usually occurs about 4 to 6 weeks prior to the time of normal lambing with abortions reaching 70% in some instances. A small percentage of ewes may die with lesions of necrosis in the maternal cotyledons. In contrast to the calf, the fetal lamb frequently develops marked lesions of focal hepatic necrosis. Ewes that have once aborted from vibriosis do not repeat the event on subsequent breedings. Infertility is not considered an important aspect of vibriosis in sheep.

## 2. Diagnosis and Control

When the herd history suggests vibriosis, the chief means of diagnosis are bacteriological recovery of the organism and immunological tests. Recovery of the organism is frequently difficult because *Campylobacter fetus* grows slowly and many of the tissues submitted for examination are grossly contaminated with bacteria that rapidly overgrow the cultures. Fetal stomach fluid, semen from the male, and fluids from the female genital tract are most frequently cultured. The fluorescent antibody technique to visualize the organism in preputial samples may be useful.

It is necessary to differentiate *Campylobacter fetus* from organisms of no pathogenic significance since there are many spiral-shaped organisms free in nature that resemble this agent. Not infrequently, the bacteriologist encounters *Campylobacter bubulus* from the genitalia of cattle, which is not considered to be pathogenic. Recent studies indicate the importance of separating the two subspecies of *Campylobacter fetus* (8, p. 125).

The infectious process appears to be localized within the bovine genital tract, and antibodies accumulate in that site. Tests for antibodies in vaginal mucus have been recognized as the best method for determining infection by the presence of antibodies. Greater sensitivity of the procedure is apparently obtained by adsorbing soluble antigens of the organism on erythrocytes or latex particles, which results in the agglutination of the particles rather than the more conventional agglutination of bacterial cells (46). It is advised that vaginal mucus samples be collected during diestrus and that agglutination tests be performed on a number of animals to establish a herd diagnosis.

The male can transmit the infection either by natural service or in semen used for artificial insemination. Successful treatment of bulls by the use of topical antibiotics has been reported, but difficulties in isolating the organism from the male genital tract always leave doubt regarding the success of the therapy. A valuable routine preventive is the addition of antibiotics to semen samples prior to their use for artificial insemination (42). Systemic administration of antibiotics to bulls may also be of value.

In most cases the infection is self-limiting in female cattle and sheep. If infected cows are given several months of sexual rest, it may be assumed that the resistance mechanism of the host will eliminate the bacterium. The tissue sterilization may be enhanced by antibiotic therapy of females with tetracyclines. The infection may be eliminated from the herd by using artificial insemination with noninfected semen for a 2-year period.

Killed vaccines are now recommended for the prevention of vibriosis. The appropriate subspecies of *Campylobacter* should be used to make the immunizing preparation, depending upon the need to protect cattle or



sheep. The bovine vaccine should be used 2 months prior to the breeding season while the ovine vaccine may be given prior to breeding or early in gestation. Recent studies suggest that the vaccine may be useful in curing persistent venereal vibriosis in cows (63). Antigenic variation of *Campylobacter fetus in vivo* has been described, which may explain irregularities encountered in serological tests and variable results from immunization with this bacterium (14).

## C. LEPTOSPIROSIS

### 1. The Disease

There are many serotypes in the genus *Leptospira* which cause diseases in domestic animals. *Leptospira pomona* is of greatest importance as a factor in reproductive failure where it affects cattle most often, but also is a significant problem in swine and horses. In cattle, this infection produces an acute phase during which animals of varied ages and both sexes may demonstrate high fevers (105°–107°F) with accompanied depression and anorexia. This stage of the infection is associated with rapid lysis of erythrocytes resulting in the expected effects of icterus, hemoglobinuria, edema, and anoxia. Mortalities occur at this stage of the disease, with up to 33% calf losses in some cases. Mortality among adult cattle is considerably less but lactating cows may have a sudden cessation of milk secretion.

It is during the convalescent period that abortions occur. This important sequel to acute leptospirosis is seen 7–10 days after the early phase and occurs at a time when antibody response is high or near its peak. The incidence varies from a few abortions up to 40% (60, p. 112). Abortions most often occur in the last one-third of pregnancy but fetuses of any age may be infected. The leptospires cross the placenta from maternal blood to fetal villi beneath the chorionic epithelium. The passage may be facilitated in advanced gestation when hemorrhage occurs at the hylus of the placentome (65). Multiplication of the bacterium is followed by fetal invasion and death. Autolysis of the fetus and the leptospires progresses rapidly. Autolytic changes are delayed in the fetal cotyledons, which prolongs the placental attachment and, thus, when abortion does occur the dead fetus is extensively autolyzed. The act of aborting the fetus seems to have little ill effect on the dam, and the fetal membranes are usually expelled naturally.

In the case of swine, the leptospires can be found in aborted pigs and in infected pigs that are weak at birth and die in a few days. Clinical disease and the act of abortion are less frequently observed in swine than in cattle. Leptospirosis occasionally causes abortions in mares from the seventh

month of gestation and later. It should be kept in mind from the standpoint of human health that urine from infected cattle, swine, and horses is a potential hazard.

## 2. Diagnosis and Control

The leptospire is delicate, spiral-shaped organisms which are sensitive to factors such as desiccation, heat, and cold. Consequently, they perish rapidly after excretion from the infected host. A chronic carrier state becomes established in many animals wherein the organism usually resides in the kidneys: This permits exit of the parasite from the host in voided urine. Swine are thought to be the most important domestic animal species in the epizootiological chain, although cattle also have been observed to shed the organism for as long as 3 months following an acute attack.

Sanitation is an important means of control. Swine should not be permitted to mingle with cattle and efforts should be made to avoid surface water, ponds, and slow moving streams if there is reason to suspect that they could be contaminated with recently voided leptospire from livestock or feral animals.

Serological tests in affected herds will demonstrate a percentage of animals reacting positively. There may be justification for some reservation about the diagnosis based on serological evidence if a clinical episode resembling the acute phase has not been a part of the disease pattern. There is value in taking a repeat reading a few weeks following the initial blood test to determine whether animals that were formerly negative are developing titers and whether the titers are rising in animals previously positive. Serodiagnosis is performed by several modifications of the agglutination phenomenon or by complement fixation. The test most commonly used is called the "agglutination-lysis test."

Dark field microscopy is also of value for obtaining prompt, presumptive diagnoses. It requires good timing to sample the animal when the organisms are in either the blood or the urine. Experience is required to differentiate the organism from artifacts.

The diagnostician is most satisfied if the organism can be recovered from the infected animal. This is an involved procedure in leptospirosis since it requires that samples be taken at the appropriate stage in the disease process and transferred promptly to media or indicator animals, because the organisms die rapidly outside the host.

Immunization of cattle, swine, and horses with a vaccine prepared from inactivated *L. pomona* is now widely practiced. Killed bacterial vaccines (bacterins) prepared from some additional *Leptospira* serotypes are also available. Unfortunately, the period of increased resistance from the use of

such vaccines is usually less than 1 year. Repeated use of these vaccines in cattle sometimes leads to anaphylactic reactions. The use of live avirulent vaccines shows promise for increasing the longevity of the resistant state (66). The reduction or elimination of the carrier state by antibiotic treatment may also be used (8, p. 494).

## D. LISTERIOSIS

### 1. The Disease

Many species of mammals and birds throughout the world have been found naturally infected with *Listeria monocytogenes*. Similar disease syndromes from the bacterium may develop in man, cattle, and sheep. Since this organism frequently invades the brain and meninges, its effects on the central nervous system have received more attention than the genital disease aspects of the infection.

Among cattle, the recorded cases that abort are usually a small percentage of the animals in a herd. Many of the abortions occur between the 4th and 7th months of gestation, with evidence that the fetus had died *in utero* before the abortion. Fetuses may present multiple foci of necrosis in the liver and sometimes in other organs such as the spleen, kidney, and lung. The organism can be obtained from these organs and from the stomach contents. Pregnant ruminants have been experimentally infected by adding *L. monocytogenes* to the drinking water, showing that ingestion is probably an important mode of transmission (24). The interesting predilection for sites of placental attachment is observed here, as in brucellosis. The *Listeria* organism actively invades the fetal tissues, causing pathological responses in them (38). When infected calves are carried to term or nearly to term, they may be born weak and die in a few days (53). The uterus of the cow usually frees itself of the organism quite rapidly following expulsion of the fetus (54).

*Listeria* encephalitis and abortion are seen occasionally in the same animal. However, the more typical observation in an infected herd is a syndrome of either encephalitis or abortion. Following abortion, cows usually recover rapidly from the associated metritis.

*Listeria* abortions in sheep occur more commonly than incidence records would indicate. Abortion rates from 1 to 50% have been recorded from such diverse areas as Australia, England, and the United States (64).

### 2. Diagnosis and Control

Isolation of the organism is the most reliable means of diagnosis. It has been found that primary culturing of infected tissues frequently fails to

disclose the organism. When tissues are held in the refrigerator for a period of several weeks, the bacterium can often be recovered. This reculture phenomenon is related in part to an unusual ability to multiply at 4°C (24, 51).

Serodiagnosis has been difficult to apply because nearly all normal adult ruminants have serum antibodies reactive with *Listeria* antigens. These have been shown to be IgM antibodies (19 S or high molecular weight antibodies). Following antigenic stimulation with *L. monocytogenes*, IgG antibodies (7 S antibodies) are also synthesized. Tests for this latter, more significant, population of antibodies can be performed by adding reducing substances to the serum, which depolymerize the IgM antibody. This finding now makes it possible to use serology diagnostically and for epidemiological investigations (52).

Acquired resistance following vaccination has been shown experimentally, but reliable vaccines are not yet available for routine use. As in brucellosis, the mechanisms of immunity are complex, involving cellular immunity, which is obtained only by immunization with live organisms (55). A reliable live attenuated strain is needed for vaccine preparation. The tetracycline group of antibiotics is indicated for therapy when the dam is clinically affected from abortion, and in the diseased newborn.

The public health aspect of this disease makes it necessary to disinfect everything contaminated by infected animals. Human cases may follow the handling of aborted animals. The isolation of the organism from milk suggests a possible additional means whereby listeriosis may be transmitted to man (12).

#### E. EPIDIDYMITIS IN RAMS

The etiological agent of this disease is a gram-negative bacterium. Studies in Australia, New Zealand, and the United States have led to its classification as a special member of the *Brucella* genus, *B. ovis* (11, 17). The infection is characterized by epididymitis in rams, but it also causes placental lesions in ewes. Lesions in the male develop initially in the tail of the epididymitis and in the interstitial tissue of the seminal vesicles and ampullae. This is followed by extravasation of sperm through the damaged epithelium. Investigators have been puzzled by the selective tissue pathogenicity shown by this organism since experiments demonstrate that the process is systemic at one stage, allowing wide tissue distribution of the organism (6). Sterility of the ram may result, although this is not necessarily complete since the process commonly involves only one testicle. Ewes may abort late in the gestation period but this is not thought to be a common process in the infection. In the gravid uterus the organism pre-

entially localizes in the interplacental region (48). This can lead to placental necrosis, separation from the caruncles, and a copious yellow exudate.

Gross lesions in the male can be detected by palpation of the scrotum. However, the presence of clinical epididymitis is an unreliable guide to the presence of *Brucella ovis* infection in individual rams (28). Serological tests including complement fixation and other procedures should be used (5, 30).

Control is based on diagnosis of the infection in rams and their elimination from the herd. Young rams should be isolated from mature rams. The immature rams may be vaccinated with two doses of a killed *B. ovis* vaccine. While there is evidence that this will increase host resistance, it must also be anticipated that antibody responses to the vaccine can lead to later difficulties in the interpretation of diagnostic serological tests (59).

### III. Virus Infections

#### A. VIRUS-INDUCED ABORTION IN HORSES

##### 1. Abortion from Equine Rhinopneumonitis Virus Infection

The equine rhinopneumonitis virus (equine herpesvirus 1) causes respiratory disease in young horses and is also the major viral cause of equine virus abortion. In 1933, Dimock and Edwards described an epizootic form of abortion in mares in Kentucky and showed that the disease was caused by a virus. This work was later confirmed in many states and several European countries. Eight years later, a report from Hungary called attention to the clinical relationship of what they regarded as "influenza" virus with equine virus abortion. They demonstrated that a mild respiratory illness followed installation of virus-infected fetal tissue suspensions on the respiratory mucous membranes. Subsequent investigations have shown that the abortion virus of Dimock and Edwards is a common respiratory tract inhabitant of the horse and that abortions may be considered a secondary effect from an agent that usually produces respiratory disease (18).

Nasal discharge, fever, depression, and cough are observed in the primary infection. Secondary bacterial infections may follow, causing the nasal discharge to become mucopurulent. Equine rhinopneumonitis virus seems to be spread by aerosols and primarily affects suckling or weanling foals. Immunity following the primary attack is transient. If the virus reinfects some months after the primary infection, the antibody response

is accelerated and only a mild form of the respiratory disease develops. Young horses may be subject to several repeated infections, which tend to enhance resistance by antigenic stimulation.

Infected pregnant mares may abort if they have not had a recent exposure to the virus. The fetus dies *in utero* and is promptly expelled without evidence of autolysis. Most abortions occur from the 8th month to term. The mare shows no signs of illness, the fetal membranes are not retained, and the animal is able to breed normally following the abortion. Some foals are born alive, but they usually die within 36 hours. The virus infection may result in the abortion of only a few mares in a group, but abortion rates can be as high as 80%.

There are widely disseminated evidences of virus infection in the fetus. Characteristic intranuclear inclusion bodies may be found in the liver, lung, spleen, lymph nodes, and the epithelium of the turbinatc bones. Commonly observed gross lesions are multiple focal areas of necrosis in the liver and edema in the lungs.

Diagnosis is dependent upon the clinical history and the detection of characteristic lesions in aborted fetuses. Tissues should be examined histologically to demonstrate the intranuclear inclusion bodies. Virus isolation can be done in investigative situations (61).

A control program has been described by Bryans (9) as a system of planned infection for use on breeding farms where the disease has been encountered. A hamster-adapted, virulent virus in lyophilized form is used to expose all horses on the premises twice yearly, in July and October. The virus suspension is administered to the nasal mucous membrane by spraying through a special catheter inserted into a nostril. Exposures are timed so that no mares are infected after the fifth month of pregnancy. The merits of the control method have been demonstrated, but a safer method of vaccination is still sought to avoid contagion to unexposed horses and to eliminate the hazard of inducing abortions with virulent virus (58).

## 2. Abortion Associated with Equine Arteritis Virus

Although equine arteritis virus (EA) was probably a common infection in earlier times, its viral etiology was not shown until 1953 (18). Outbreaks of the disease occur sporadically in the United States. The disease is reported uncommonly in other parts of the world. The agent is now provisionally classified in the togavirus group (27). The horse is the only known susceptible animal and only one immunogenic virus type is known to exist.

The characteristic lesion of this disease is an inflammatory reaction in

small arteries. These lesions can occur in widely distributed parts of the body and their secondary effects give rise to the characteristic forms of the disease. The "pulmonary" form is accompanied by pulmonary edema, emphysema, and large quantities of serofibrinous effusion in the pleural cavity. An animal thus affected shows pulmonary distress as well as edema of the limbs and swelling around the eyes. The abdominal or "typhoid fever" form results from infarction in mesenteric vessels and, secondarily, edema in the submucosa of the intestine. Mild or severe colic accompanied by a watery diarrhea may be seen in such cases. Adult horses suffering from this infection are depressed and show muscular weakness. Death may occur but this is probably a rare result from the natural infection.

The interesting vascular lesions of this disease probably result from complexes of antigen (virus) and antibody that form in the walls of arteries. The lesions resemble those seen in various immunological diseases of man and animals where the infectious etiology is still uncertain (15).

Abortions associated with this infection may affect 50-80% of the mares. The abortions tend to occur in the latter half of the gestation period. The tissues and the fluids of infected, aborted fetuses contain large quantities of virus. Since the disease is spread by aerosols and contracted by inhalation, horses with signs of arteritis should be isolated for several weeks (41). A modified live virus vaccine has been developed by repeated passage of the virus in tissue cultures of horse kidney cells and rabbit kidney cells (40). Commercial availability of the vaccine will apparently depend upon the prevalence of the disease and the need for immunization.

## B. VIRUS-INDUCED ABORTION IN CATTLE

### 1. Infectious Bovine Rhinotracheitis-Infectious Pustular Vulvovaginitis Virus Infection

The virus causing this disease is responsible for several important syndromes in cattle (8, p. 968). The infection is transmitted by contact of the virus with mucous membranes. Infectious bovine rhinotracheitis (IBR) follows exposure of the respiratory tract of susceptible cattle to this agent of the herpesvirus group.

When the genital tract is exposed, either by venereal transmission or contaminated instruments, such as insemination pipettes, infectious pustular vulvovaginitis (IPV) develops in the cows, and infectious pustular balanoposthitis appears in bulls. In the females circumscribed pustules appear over the lymphatic follicles in the vulva. When the pustules coalesce, a purulent exudate accumulates as a sticky discharge on the outside of the

vulva. This disease may spread rapidly through a herd and usually begins with febrile reactions. During its acute stage of 10 to 14 days, the cows have considerable pain in the affected tissues and urinate frequently. Temporary infertility may occur and breeding should be suspended during this early phase of the process. Special measures may be necessary to avoid adhesions of the preputial tissues in affected bulls. Diagnosis is made by isolation of the virus and serum neutralization tests.

Unfortunately, the transient disease that the cow experiences can result in viral invasion of the fetus causing a generalized necrotizing process with death and abortion during the latter half of the gestation period (36). The dead and autolyzed fetuses are aborted about 3 weeks to 3 months following infection of the dam. The placenta is often retained, although placental lesions are not dominant (35).

Immunization of cattle with a modified live virus vaccine is now widely employed for the prevention of both infectious bovine rhinotracheitis and infectious pustular vulvovaginitis (68). Good control of the disease is obtained when the vaccine is administered at approximately 6 months of age, and to nonpregnant adult females. Abortions are likely to follow if pregnant cattle are vaccinated.

## 2. Bovine Viral Diarrhea-Mucosal Disease Virus Infection

The virus of bovine viral diarrhea-mucosal disease (BVD-MD) infects a high percentage of the cattle in the United States and other parts of the world. In many cattle populations it is common to find that approximately one-half of the animals have serum antibodies against this agent of the togavirus group (27). Mucosal disease is the severest form of the infection, resulting in ulcerations in the oral cavity and throughout the mucous membrane of the digestive tract (8, p. 1283). In such cases, the animals die without developing an effective immune response. The more common form of bovine viral diarrhea is a less severe process producing diarrhea and debility. In pregnant cows the virus may invade the fetuses to induce either abortions or teratological effects. The problem of fetal deformity is discussed in Section III, D dealing with viruses that affect fetal development. Abortions are most apt to occur in the first trimester of pregnancy (34). The dead fetuses are usually aborted, but in some cases they undergo mummification in the uterus. In the study of a natural outbreak, Kahrs *et al.* (33) found that 34% of the pregnancies were abnormal, but the usual incidence of abortion from this infection is thought to be relatively low. Diagnosis of BVD-MD abortion is difficult because it may not be possible to isolate the virus from the dead fetus, which is commonly held in the uterus for many days prior to abortion. Since abortion occurs early in



the gestation period, the aborted fetus may not be observed and one may be led erroneously to consider infertility as the basis of a herd problem.

The diseases caused by BVD-MD virus are effectively prevented by immunization with a modified live virus vaccine. Pregnant animals should not be vaccinated. Immunization is commonly performed when the cattle are 8-12 months of age.

### 3. Epizootic Bovine Abortion

This disease (EBA) is encountered in cattle that graze on foothill and mountain areas of the far western United States, especially California. A viral etiology is thought to be most likely, but the agent has not yet been isolated. Chlamydial organisms have been isolated from some aborted fetuses but this is an irregular finding and their etiological role is uncertain (44).

The disease primarily affects beef cattle in the last trimester of pregnancy with abortions occurring in the fall and winter months. In susceptible herds, females of all ages may abort, but in succeeding years the losses are limited to heifers in their first gestation. Abortion rates of 80% have been encountered. The cows do not show systemic effects from the disease and their fertility is unimpaired following the abortion.

Gross pathological changes in the fetus include widely disseminated petechial hemorrhages and reticuloendothelial hyperplasia in liver, lymph nodes, and spleen. Some of the fetuses have an enlarged liver that is coarsely granular and friable. Histologically, the affected livers show granulomatous inflammation with massive infiltration of macrophages and extensive loss of glandular parenchyma (31).

At the present time, there is no basis for the control of this disease. The disease syndrome has followed the feeding of *Ornithodoros coriaceus* ticks on cattle during the first half of pregnancy, which implies a vector role for the arthropod (62). The clinical observation that cows characteristically abort only once suggests that successful immunization might easily be accomplished once the causative agent has been cultivated *in vitro*.

### C. ENZOOTIC ABORTION OF EWES

The synonym for this disease is ovine virus abortion. The etiological agent is an obligate intracellular parasite, *Chlamydia psittaci*, which has some viruslike characteristics but is not actually a virus. Within infected host cells the coccoid elementary bodies of the organism form microcolonies in cytoplasmic vesicles. Strains of the organism cause diverse diseases including psittacosis and ornithosis in birds (10). The strain

associated with placentitis leading to abortion in sheep was first reported in 1950 by Stamp *et al.* (67) in Scotland. Since that time the disease has been reported from several countries in Europe and many parts of the United States (8, p. 788).

Abortions and premature lambing occur during the last month of gestation. Retention of the placenta and vaginal discharge commonly follow the act of abortion. Ewes may be visibly ill with some febrile response for a period of a few days. Those that do not expel the dead fetuses may die. In a susceptible group of animals, the abortion rate may be as high as 30%, but is more often 5% or less (47). Fertility of the ewes is not affected on subsequent breedings, and rams exposed to the organism appear to remain uninfected.

Clinically this disease resembles vibriosis, and it is necessary to eliminate *Campylobacter fetus* as a factor in the epizootic. Smears prepared from infected placentas can be used to demonstrate characteristic elementary bodies on microscopic examination. In addition, infected placental tissue may be used for isolation of the agent in embryonated chicken eggs. Tests for complement-fixing antibodies will give an indication of the morbidity rate within the population of affected animals, but it must be remembered that the test is not specific for the organism of enzootic abortion of ewes (56). A vaccine prepared in England by formalin inactivation of the organism cultivated in embryonated eggs has been used rather extensively in Europe and the United States (22). This is reported to induce a resistant state lasting more than 2 years.

#### D. VIRUSES AFFECTING FETAL DEVELOPMENT

The association of abnormalities in the newborn with infections sustained by the pregnant female was given worldwide attention in 1941 in a report by Gregg of Australia (25). Following an unusually severe rubella (German measles) outbreak numerous cases of malformed infants were found among babies that had been born to mothers infected with rubella virus during the first 3 months of gestation. The virus infection had caused cataracts, deafness, heart disease, and microcephaly in the infants.

Immunization against virus diseases is frequently accomplished by inoculating attenuated live viruses. Their use establishes a mild form of the virus infection at a time selected as appropriate for induction of the resistant state. In certain animal diseases, these immunization practices can result in diseases of the fetus similar to those following rubella infection in women.

Bluetongue, a systemic virus disease of sheep, may be prevented by immunization with such a modified live virus vaccine. If ewes are im-

immunized around the 5th or 6th week of gestation, the virus crosses the placenta and infects the fetus producing cytopathic events in various sites, including the brain. Since the central nervous tissue has little capacity for regeneration, the lambs are born with congenital cavitory anomalies of the brain (hydranencephaly and porencephaly). Some lambs are stillborn while others carried to term may be "dummies" that make no effort to nurse. There is extensive loss of cerebral and cerebellar tissue as well as retinal dysplasia (50). Lamb losses may average 5% and reach 50% in some groups.

When pregnant sows are immunized against hog cholera with the modified live virus vaccine, the baby pigs may develop abnormally with cerebellar hypoplasia and congenital tremors. Less common effects are mummification, contracted tendons, brachygnathia, and ascites. Vaccine virus can be detected in the newborn pigs (20).

Immunization of pregnant cattle with the modified live virus vaccine for bovine viral diarrhea-mucosal disease, or the natural infection, may induce calf losses from cerebellar hypoplasia, mummification, and stillbirths. Ocular defects, alopecia, and brachygnathia may be seen in the calves (32).

Natural infection with the infectious bovine rhinotracheitis virus and vaccination with the modified live virus vaccine were associated with fetal invasion by the virus and the production of necrotizing lesions in the fetal liver and the placenta (37).

It is difficult to estimate the total effect of animal losses due to these wasted pregnancies resulting from both natural infection and immunization. Judicious use of live virus vaccines is the best means of avoiding such reproductive losses.

#### IV. Protozoan Infections

##### A. TRICHOMONIASIS

###### 1. The Disease

Trichomoniasis is a protozoan infection of the genital tract which is transmitted by the bull. In the female it causes death of the fetus, abortion, pyometra, and sterility. The organism, *Trichomonas foetus*, has three anterior flagella and is 10–25  $\mu$ m in length. It has an undulating membrane the length of the cell which is kept in constant motion as it moves through extracellular fluids (39, p. 89). The organism persists in the prepuce of infected bulls for years. When infected bulls breed the cows, or are used for artificial insemination, the disease may become widespread in a herd.

Pregnancy following such a mating frequently terminates in death of the fetus in the first trimester. The fetus may then decompose in an accumulation of pus and fluid in the uterus. Maceration of the fetus in these fluids may continue for several months, or the uterus may expel an early embryo, permitting the cow to return to estrus. The discharge of exudates is frequently observed in an infected herd. Physical examination will reveal the discharge on the floor of the vagina or exuding from the uterus (2).

## 2. Diagnosis and Control

The infection is diagnosed by demonstrating the organism in exudates from the female or in preputial washings from the male. Care must be exercised to differentiate *T. foetus* from other flagellates that may be encountered. When samples are collected from the cow at a favorable time, the organisms can be seen in great numbers from fresh, unstained preparations. Demonstration of *T. foetus* in genital washings from the male is usually difficult. Negative results should not be considered proof of freedom from infection. A more reliable indicator of the infection status of the bull can be obtained by the examination of vaginal samples from virgin heifers 12–20 days following natural service.

As soon as the disease is recognized in a herd, sexual rest should be instituted since the organisms do not remain permanently in the genital tract of the female. After the infectious contents have been voided from the uterus, estrus will become reestablished and the trichomonads will be eliminated from the genital tract. The females may be bred by uninfected males 1–2 months after all discharge has ceased. Ordinarily, infected bulls should be eliminated from the herd. However, Bartlett (1) has reported success in eliminating the organism from infected bulls with topical treatment. This procedure is complicated and time-consuming. Its application is limited to valuable sires. Successful systemic treatment of infected bulls by the oral administration of dimetridazole has been described (45). When applicable to the management of the herd, artificial insemination from a carefully supervised breeding service should be used.

## B. TOXOPLASMOSIS

### 1. The Disease

Toxoplasmosis in man and animals is caused by a protozoan, *Toxoplasma gondii*. The parasite is a small, elongated organism (4–7  $\mu\text{m}$ ), tapered at both ends, and frequently seen in compact masses or cystlike structures (39, p. 294). The organism is now recognized to be a member

from these defects may appear normal at birth, but their deficient lymphoid systems deprive them of normal resistance mechanisms to microbial agents in the extrauterine environment. The animals are destined to experience early death from uncontrolled infectious processes. The problem is best exemplified in horses, where some foals of the Arabian breed have been found to inherit a combined immunodeficiency state (43). This is combined in the sense that deficiencies of both antibody production and cell-mediated immunity are present. The foals are highly susceptible to both viral and bacterial infections, and they usually die within 4 months of age from a complex of adenovirus and bacterial pneumonitis. The condition is assumed to be inherited as an autosomal recessive trait.

A primary immunodeficiency syndrome has been reported in cattle of the Black Pied Danish breed (7). Calves descended from a bull carrying the recessive gene were deficient in thymus-derived lymphocytes. This deprived the calves of their cell-mediated immunological defenses and also blunted the capacity to produce antibodies to many antigens. The calves experienced a variety of progressive infections which usually ended fatally. Heritable immunodeficiencies are also known to occur in man and in some laboratory animals (4). It may be anticipated that this problem will be recognized with increasing frequency as the appropriate diagnostic tests become applied more commonly.

## B. SPORADIC CAUSES OF GENITAL INFECTIONS

Space permits little more than mention of several organisms that are associated occasionally with reproductive deficiency. Some organisms which may be derived from the intestinal tract may be involved in abortions, such as *Escherichia coli*, streptococci, and staphylococci in several species of animals, and *Actinobacillus equuli* in solipeds. *Salmonella abortusovis* affecting sheep in Europe and *Salmonella abortusequi* in horses are two more organisms of the enteric group that occasionally cause abortions (8).

Other incriminated bacteria have been *Pseudomonas aeruginosa* and *Corynebacterium equi*. *Corynebacterium pyogenes* is responsible for a wide variety of suppurative processes in various species of mammals. In the reproductive tract, it is commonly present in purulent metritis.

An organism named *Actinobacillus seminis* has been described as causing epididymitis in rams. *Mycoplasma bovis genitalium* may be isolated from the genitals of both cows and bulls. In experimental infections the organism produces epididymitis (57).

Certain fungi, such as *Aspergillus fumigatus* and *Absidia ramosa*, invade the placental tissues and also produce lesions in the fetus (69).

Genital disease is a major effect of dourine (*Trypanosoma equiperdum*) in horses, and *Babesia caballi*, the agent of equine piroplasmiasis, has been found in aborted foals (8)

There is increasing concern about the role of viruses as factors in embryo mortality and abortion Dunne *et al* (19) have described porcine reproductive failure (embryonic death and infertility) associated with a picornavirus. Some additional viruses associated with reports of abortion or teratogenic effects are the Japanese-B encephalitis virus in swine, Wesselsbron virus in sheep, Rift Valley Fever virus in several species of ruminants, and the pseudo-rabies virus in swine (Aujeszky's disease) (21)

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